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(71) Applicant: MONSANTO COMPANY [US/US]; 800 North Lindbergh Blvd., St. Louis, MO 63167 (US).

(72) Inventors: BOROKOV, Alexandre, Y.; 13279 Amiot Dr., St. Louis, MO 63146 (US). VALENTIN, Henry, E.; 873 M Fox Spring Dr., Chesterfield, MO 63017 (US).

(74) Agent: KAMMERER, Patricia, A.; Howrey Simon Arnold & White, LLP, 750 Bering Drive, Houston, TX 77057-2198 (US).

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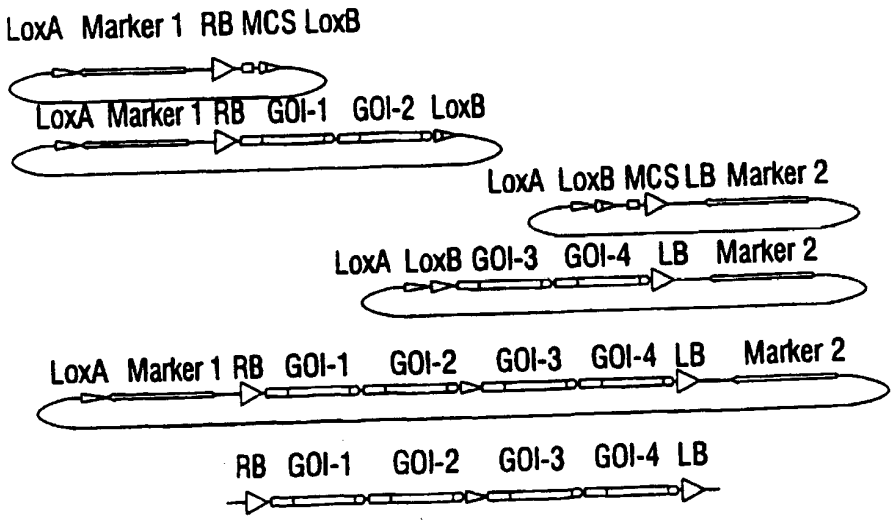

(57) Abstract: The present invention relates to a novel unconventional method for cloning large and multiple segments of DNA into a vector. More specifically the present invention provides nucleic acid sequences for selectively regulating site-specific recombination in favor of insertion of multiple segments of DNA in a plant transformation vector. In particular, the invention relates to the use of *sequences* in the recombination site that can be used in gene-stacking or other multigenic cloning strategies.

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NOVEL CLONING METHODS AND VECTORS

FIELD OF THE INVENTION

The present invention relates to molecular biology. More specifically, the present invention relates to the design and use of novel cloning vectors for introducing large and multiple segments of DNA into plants.

BACKGROUND OF THE INVENTION

The tools of molecular biology have enabled researchers to introduce segments of DNA from one organism to another organism. Recombinant DNA technology has positively impacted the areas of medicine and agriculture. Conventional cloning methods have enabled the introduction of new pharmaceuticals and improved crops of agricultural importance. As the need for the introduction of multiple segments of DNA and larger fragments of DNA into numerous target hosts increases, the need for novel cloning strategies increases accordingly.

Typical recombinant DNA techniques involve a range of methods for isolating, analyzing and manipulating the DNA *in vitro* prior to introducing the DNA into a target host. First the DNA of the donor vector or donor genome is prepared. In a basic approach, restriction enzymes also referred to as restriction endonucleases are used to cut the donor DNA at specific locations. Different restriction enzymes recognize different sequences of nucleotides on the DNA and cleave the DNA polymer at these sequences. DNA segments are then isolated and ligated into a cloning vector. The ligation process relies on the use of another enzyme (DNA ligase) that can bond segments of DNA together. A cloning vector is a nucleic acid molecule into which DNA fragments can be introduced *in vitro* using the restriction enzymes and DNA ligases. A number of cloning vectors exist including but not limited to plasmid and bacteriophage cloning vectors. Different types of cloning vectors can be used, depending on the target host organism into which the DNA is introduced.

One of the limitations of conventional cloning strategies is the need to isolate and purify the DNA and the use of restriction enzymes in the cloning process. Introducing large DNA fragments and multiple DNA fragments is also difficult. For example, large DNA fragments are often difficult to clone because the fragments contain internal restriction sites that limits the number of usable restriction enzymes for the cloning process. Thus, special vectors must be designed and rare restriction enzymes may be necessary to clone large segments of DNA. Also, to introduce multiple segments of DNA into a cloning vector requires the isolation and

purification of each segment of DNA prior to the ligation step and subsequent screening to find the desired cloning product. Consequently, a novel cloning strategy that reduces the need for multiple isolation and purification steps, that does not solely rely on the use of restriction enzymes, and that can be used to clone any size DNA fragment would be more efficient, less labor intensive, and an improvement over existing cloning methods.

Recombination generally refers to the joining of nucleic acids either biologically or through laboratory methods. Homologous recombination can occur between DNA sequences that have nucleotide sequences in common. Site-specific recombination refers to recombination that occurs at specific regions that is regulated by specific enzymes known as site-specific recombinases. These recombinases recognize short stretches of DNA and a cross-over or physical exchange of DNA can take place in the presence of the recombinase.

Many site-specific recombination systems exist. One such system is known as the Cre/lox site-specific recombination system from *E. coli* bacteriophage P1 (Sternberg et al., J. Mol. Biol. 187:197-212, 1986). The Cre recombinase recognizes a 34 base pair *lox* sequence that contains two 13 base pair recombinase recognition/inverted repeat sequences. The recombinase recognition/inverted repeat sequence refers to two identical copies of a sequence of bases of DNA on the same DNA molecule that are repeated in reverse orientation but on opposite DNA strands in a double-stranded DNA molecule. The two inverted repeats are separated by an asymmetric eight base pair sequence that defines orientation of the site, also known as the core or spacer sequence region. Four molecules of Cre recombinase will recognize and bind to a pair of *lox* sequences (one per inverted repeat) and catalyze a reciprocal cross-over between these sequences. If the asymmetric *lox* sequences are directly repeated, the DNA between the *lox* sequences will be excised. If the *lox* sequences are in an inverted orientation, the DNA between the *lox* sequences will be inverted. The recombinase reaction is a reversible reaction, the product can be an excised DNA segment or a recombined DNA product.

Site-specific recombination can be used as a tool for cloning purposes, because site-specific recombination in essence can replace the restriction, purification, and ligation steps of conventional cloning in an efficient cross-over process. A number of manufacturers have developed cloning kits that are based on the use of site-specific recombination systems (see, for example Novagen (Madison, WI), CLONTECH (Palo Alto, CA), Invitrogen (Carlsbad, CA), and BRL Life Technologies (Rockville, MD). Site-specific recombination systems have also been

investigated in plant systems for activating genes (Odell et al., Plant Physiol., 106:447-458, 1994), investigating chromosomal rearrangements (Medberry et al., Nucl. Acids Res. 23:485-490, 1995), and for excising or removing genes (Yoder and Goldsborough, Bio/Technology 12, 3:263-267, 1994; Ow and Dale, Miami Short Rep., 2:6, 1992). A cloning system that uses the Cre/lox recombination system has been developed for cloning of large DNA segments, this system uses site-specific recombination to position the recombinant DNA into a context for expression of a reporter gene (Liu et al., Current Biology 8:1300-1309, 1998). The site-specific recombination systems that have been developed for use in plants are predominantly focused on excision mechanisms using the wild-type recombinase recognition sequences. One limitation of the use of wild-type recombination recognition sequences is that the recombination reaction is reversible and is shifted in the favor of excision of DNA over insertion of DNA. In other words, for cloning purposes, the stability of the inserted fragment(s) into a cloning vector is important, and a system that allows the inserted DNA to excise easily would be undesirable. More preferably, a system that is designed to favor the insertion process that also allows for the insertion of large or successive multiple segments of DNA into a single vector would be desirable in the art.

The biological properties of the Cre/lox site-specific recombination system have been studied. Hoess et al. (Nucl. Acids Res. 14:2287-2300, 1986) disclosed that a recognition sequence that efficiently recombines with itself, but not with the wild-type recombination sequence can be generated by modification of the spacer region of the lox sequence. Lee and Saito (Gene, 216:55-65, 1998; WO99/25851) disclosed that specific changes of nucleotides in the spacer region affected the biological activity and specificity of the recognition site.

The Cre recombinase has a high affinity for the target recombination site (*loxP*), the inverted repeats of the lox sequence (Ringrose et al., J. Mol. Bio., 284:363-384, 1998; Hoess and Abremski, Proc. Natl. Acad. Sci. USA, 81:1026-1029, 1984). The inverted repeats, including the regions distant from the spacer region can be significantly modified without a noticeable effect on the binding properties (Hartung and Kisters-Woike, J. Biol. Chem., 273:22884-22891, 1998). Albert et al. (Plant J., 7:649-659) disclosed that sequences containing only six unmodified nucleotides of the 13 total nucleotides can still recombine and that the modifications can effect the rates of forward and reverse recombination reactions.

One of the goals of plant genetic engineering is to produce plants with agronomically important characteristics or traits. Recent advances in genetic engineering have provided the requisite tools to transform plants to contain and express foreign genes (Kahl et al. (1995) World Journal of Microbiology and Biotechnology 11:449-460). Particularly desirable traits or qualities of interest for plant genetic engineering would include but are not limited to resistance to insects and other pests and disease-causing agents, tolerances to herbicides, enhanced stability, yield, or shelf-life, environmental tolerances, and nutritional enhancements. The technological advances in plant transformation and regeneration have enabled researchers to take segments of DNA, such as a gene or genes from a heterologous source, or a native source, but modified to have different or improved qualities, and incorporate the exogenous DNA into the plant's genome. The gene or gene(s) can then be expressed in the plant cell to exhibit the added characteristic(s) or trait(s). In most transformation approaches, a single vector containing 1-2 genes conferring desirable plant characteristic(s) is introduced into a target plant of interest via an appropriate expression vector. Subsequent genes are introduced either through plant breeding, or by a second transformation process using a different expression vector containing a second or third gene of interest.

Conventional strategies for introducing multiple genes into target plants of interest are time-consuming and labor intensive. For example, to introduce multiple genes of interest into a target plant requires the introduction of the plant expression vector and subsequent screening of the transformed plants for the desired characteristic(s), followed by a second transformation event into a parent plant generated by the first step, with subsequent screening of the second generation of transformed lines for the desired characteristic(s) conferred by the gene(s) on the second expression construct. This method can be a time intensive process, essentially doubling or tripling the time and labor it takes to generate a plant with desirable characteristics in a conventional cloning method using multiple purification steps and restriction enzymes. Manipulation with large fragments of DNA using conventional cloning techniques is even more challenging and thus there is a great need in the art for more efficient methods of introducing large, multigene cassettes into target plants of interest. The construction of large cloning vectors herein referred to as megavectors, over 10 kilobases is extremely inefficient using current methodologies.

A novel cloning method that allows the construction of a plant expression vector in that multiple genes can be introduced into a plant simultaneously, would be a tremendous advantage over existing methods. The utility of using non-crossreactive recognition sequences for cloning large multigenic DNA fragments is a novel cloning approach with broad applications. For example, a single round of recombination can be used to construct a plant expression vector containing multiple genes in one step. Thus, a cloning vector containing multiple modified recombination sequences can also be efficiently used in gene-stacking approaches saving time and labor.

SUMMARY OF THE INVENTION

The present invention provides recombination DNA molecules of an intregrase family site-specific recombination system designed by creating one or more mutations in the recombination site spacer sequence, the recombinase recognition/inverted repeat sequence or both sequences.

In one aspect, the method of the present invention provides modification of the *loxP* recognition sequence of the Cre/lox site-specific recombination system of SEQ ID NOS: 5-97 comprising at least one change in spacer, the inverted repeat regions or both regions. These modified sequences possess two novel features that distinguish them from the wild *loxP* site. First, the recombination can take place only between certain pairs of the modified sequences. Second, the new site, generated by recombination of such pair, becomes resistant to further recombination neither with wild *loxP* site nor with any of the modified sequences.

In another aspect, the present invention provides one or more novel DNA vectors comprising at least one modified recombination DNA of SEQ ID NOS: 5-97.

In another aspect, the present invention provides plants comprising at least two recombinant DNA molecules wherein at least one is resistant to further recombination and one is susceptible to further recombination and are selected from SEQ ID NOS: 5-97.

More specifically, the present invention provides a method of cloning segments of DNA into a vector by incorporating at least one modified recombination DNA molecule in one or more cloning vectors, recombining the vector(s) in the presence of a recombinase, and screening and/or selecting a desired recombination event.

In still another aspect, the present invention provides a method of introducing multiple DNA segments into a plant vector by incorporating multiple modified recombination DNA

molecules compatible for recombination reaction of which some recombine to create recombination resistant sequences, leaving others still capable of recombination thus allowing successive rounds of recombination to introduce additional segments of DNA.

In order to produce a transgenic plant using the novel cloning strategy of the present invention, a plant cloning vector that includes a heterologous gene sequence that confers the desired phenotype when expressed in the plant is introduced into the plant cell. The vector also includes a plant promoter that is operably linked to the heterologous gene sequence, often a promoter not normally associated with the heterologous gene. The vector also includes a 3' non-translated DNA sequence that functions in plant cells to cause the addition of polyadenylated nucleotides to the 3' end of the RNA sequence. In addition to these basic genetic elements of a typical plant expression vector including other replication, T-DNA transfer, and regulatory sequences, the novel plant vector includes one or more modified recombination DNA molecules that function as sequences for site-specific recombination systems. The vector can be constructed by one or more steps of recombination *in vitro*, and the desired recombination product is introduced into a plant cell by standard methods to produce a transformed plant cell, and the transformed plant cell is regenerated into a transgenic plant.

The foregoing and other aspects of the invention will become more apparent from the following detailed description and accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a plasmid map of pMON21659

Figure 2 is a plasmid map of pAYB200*loxP*

Figure 3 illustrates recombination products of two *loxP* modified sequences

Figure 4 illustrates a "Two into One" cloning method

Figure 5 illustrates plasmid maps of pMON38260 and pMON38252 half-vectors

Figure 6 are plasmid maps illustrating a single recombination event and a double recombination event.

Figure 7 is an illustration of a "Multiple Gene Stacking" cloning method.

Figure 8 illustrates plasmid maps of pMON38271 (binary vector), pMON38288 (shuttle I) and pMON38997 (shuttle II) for the gene stacking approach.

Figure 9 illustrates the products of recombination of the binary vector with shuttle I.

Figure 10 is a plasmid map of pMON38254.

Figure 11 is a plasmid map of pMON38264.

Figure 12 is a plasmid map of pMON38267.

Figure 13 is a plasmid map of pMON38986.

Figure 14 is a plasmid map of pMON38993.

Figure 15 is a plasmid map of pMON54017.

The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. Definitions of common terms in molecular biology may also be found in Rieger et al., Glossary of Genetics: Classical and Molecular, 5th edition, Springer-Verlag: New York (1991); and Lewin, Genes V, Oxford University Press: New York (1994). The nomenclature for DNA bases as set forth at 37 CFR § 1.822 is used. The standard one- and three-letter nomenclature for amino acid residues is used.

"CP4". "aroA:CP4 EPSPS" and "CP4 EPSPS" and "CP4" refer to the EPSP synthase gene or protein purified from *Agrobacterium tumefaciens* (AGRTU) strain CP4 that when expressed in plants confers tolerance to the herbicide glyphosate. The gene sequence maybe native or modified for enhanced expression in plants.

"DNA segment". Refers to any length of DNA sequence.

"Operably Linked". A first nucleic-acid sequence is "operably" linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a protein-coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, are in the same reading frame. DNA molecules from different sources may be physically "linked", refers to the linear location of the DNA molecules.

"Construct" or "vector" refers to any plasmid, cosmid, virus, autonomously replicating sequence, phage, or other linear or circular single-stranded or double-stranded DNA or RNA derived from any source that includes one or more DNA sequences, such as promoters, protein-coding sequences, 3' untranslated regions, etc., that have been linked in a functionally operative manner by reconbinant DNA techniques. Recombinant vectors for plant transformation are commonly double-stranded circular or linear DNA molecules, although other vector systems are

suitable for the practice of the present invention including but not limited to binary artificial chromosome (BIBAC) vectors (Hamilton et al., Gene 200:107-116 (1997)), and RNA viral vectors (Della-Cioppa et al., Ann. N.Y. Acad. Sci. 792: 57-61 (1996)), for example. Optionally, the DNA construct includes a replication system. Conventional compositions and methods for making and using recombinant nucleic acid constructs are discussed, inter alia, in Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; and Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates). See also, e.g., Mailga et al., Methods in Plant Molecular Biology, Cold Spring Harbor Press (1995); Birren et al., Genome Analysis: Detecting Genes, 1, Cold Spring Harbor, New York (1998); Birren et al., Genome Analysis: Analyzing DNA, 2, Cold Spring Harbor, New York (1998); and Clark et al., Plant Molecular Biology: A Laboratory Manual, Springer, New York (1997).

Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage and Carruthers, Tetra. Letts. 22:1859-1862 (1981), and Matteucci et al., J. Am. Chem. Soc. 103:3185 (1981). Chemical synthesis of nucleic acids can be performed, for example, on commercial automated oligonucleotide synthesizers.

A number of vectors suitable for stable transformation of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., Cloning Vectors: A Laboratory Manual (1985, supp. 1987); Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press (1989); and Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers (1990). Typically, plant expression vectors include one or more transcription units, each of which includes: a 5' untranslated region, that includes sequences that control transcription (e.g., cis-acting promoter sequences such as enhancers, the transcription initiation start site, etc.) and translation (e.g., a ribosome binding site) of an operably linked protein-coding region (i.e., a "promoter"); a protein-coding region (or "open reading frame" or ORF); a 3' untranslated region that includes additional regulatory regions from the 3' end of plant genes (Thornburg et al., Proc. Natl. Acad. Sci. USA 84:744 (1987); An et al., Plant Cell 1:115 (1989), e.g., a 3' terminator region to increase mRNA stability. In addition, such constructs commonly include a selectable or screenable marker and optionally an origin of replication or other sequences required for replication of the vector in a host cell.

Plant expression vectors optionally include RNA processing signals, e.g., introns, that may be positioned upstream or downstream of a polypeptide-encoding sequence in the transgene. In addition, the expression vectors may also include additional regulatory sequences from the 3'-untranslated region of plant genes.

"Promoter" or "promoter region" refers to a nucleic acid sequence, usually found upstream (5') to a coding sequence, that controls production of messenger RNA (mRNA). For embodiments of the invention in which the use of a constitutive promoter is desirable, any well-know constitutive plant promoter may be used. Constitutive plant promoters include, for example, the cauliflower mosaic virus (CaMV) 35S promoter, that confers constitutive, high-level expression in most plant tissues (see, e.g., Odel et al., Nature 313:810 (1985)), including monocots (see, e.g., Dekeyser et al., Plant Cell 2:591 (1990)); Terada and Shimamoto, Mol. Gen. Genet. 220:389 (1990)); the nopaline synthase promoter (An et al., Plant Physiol. 88:547 (1988)), the octopine synthase promoter (Fromm et al., Plant Cell 1:977 (1989)), cauliflower mosaic virus 19S promoter, figwort mosaic virus 35S promoter, sugarcane bacilliform virus promoter, commelina yellow mottle virus promoter, rice cytosolic triosephosphate isomerase promoter, adenine phosphoribosyltransferae promoter, rice actin 1 promoter, mannopine synthase promoter, histone promoter, and a tobacco constitutive promoter as disclosed in U.S. Patent No. 5,824,872.

For other embodiments of the invention, well-known plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals may be used, including promoters regulated by (1) heat (Callis et al., Plant Physiol. 88:965 (1988)), (2) light (e.g., pea rbcS-3A promoter, Kuhlemeier et al., Plant Cell 1:471 (1989); maize rbcS promoter, Schaffner and Sheen, Plant Cell 3:997 (1991); or chlorophyll a/b-binding protein promoter, Simpson et al., EMBO J. 4:2723 (1985)), (3) hormones, such as abscisic acid (Marcotte et al., Plant Cell 1:969 (1989)), (4) wounding (e.g., wunI, Siebertz et al., Plant Cell 1:961 (1989)); or (5) chemicals such as methyl jasmonate, salicylic acid, etc. It may also be advantageous to employ (6) organ-specific promoters (e.g., Roshal et al., EMBO J. 6:1155 (1987); Schernthaner et al., EMBO J. 7:1249 (1988)); Bustos et al., Plant Cell 1:839 (1989)).

"Selectable" or "screenable markers". A vector or construct may also include any gene that encodes a selectable marker to select for, or a screenable marker to screen for, plants or plant cells that contain the exogenous genetic material. Examples of selectable markers include,

but are not limited to: known genes encoding resistance to antibiotics such as hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin, such as the neomycin phosphotransferase (neo) gene (Potrykus et al., Mol. Gen. Genet. 199:183-188 (1985), that confers resistance to kanamycin and G418) and a dihydrofolate reductase (DHFR) gene, that confers resistance to methotrexate (Thillet et al., J. Biol. Chem. 263:12500-12508 (1988)); or known genes encoding herbicide tolerance, e.g., bar, that confers bialaphos resistance; a mutant EPSP synthase gene (Hinchee et al., Bio/Technology 6:915-922 (1988)), that confers glyphosate resistance; a nitrilase gene, that confers resistance to bromoxynil (Stalker et al., J. Biol. Chem. 263:6310-6314 (1988)); a mutant acetolactate synthase gene (ALS), that confers imidazolinone or sulphonylurea resistance (EP 154,204; etc. Examples of screenable markers include a β-glucuronidase or uidA gene (GUS), that encodes an enzyme for which various chromogenic substrates are known (Jefferson, Plant Mol. Biol, Rep. 5:387-405 (1987); Jefferson et al., EMBO J. 6:3901-3907 (1987)); an R-locus gene, that encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta et al., Stadler Symposium 11:263-282 (1988)); a β-lactamase gene (Sutcliffe et al., Proc. Natl. Acad. Sci. (U.S.A.) 75:3737-3741 (1978)); a gene that encodes an enzyme for which various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a luciferase gene (Ow et al., Science 234:856-859 (1986)); a xylE gene (Zukowsky et al., Proc. Natl. Acad. Sci. (U.S.A.) 80:1101-1105 (1983)), that encodes a catechol dioxygenase that can convert chromogenic catechols; an α-amylase gene (Ikatu et al., Bio/Technol. 8:241-242 (1990)); a tyrosinase gene (Katz et al., J. Gen. Microbiol. 129:2703-2714 (1983)), that encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone, that in turn condenses to melanin; green flourescence protein (Elliot et al., Plant cell Rep. 18:707-714 (1999)) and an α-galactosidase.

Also included are genes that encode secretable markers such as antigens that can be identified following secretion by antibody interaction (e.g., by ELISA) or enzymes that can be detected catalytically (e.g., α-amylase, β-lactamase, phosphinothricin transferase), or proteins that are inserted or trapped in the cell wall (such as proteins that include a leader sequence such as that found in the expression unit of extension or tobacco PR-S).

"Recipient vector". Refers to the DNA molecule in a site-specific recombination system that receives a DNA segment.

"Recombinase recognition/inverted repeat sequence". Refers to the DNA sequence that functions as a recombinase binding site in a site-specific recombination system.

"Recombination resistant". Refers to the status of the recombined DNA molecules of a site-specific recombination for further capacity to continue as a substrate for a recombination reaction.

"Site-specific recombination system". Any of the numerous DNA sequence and recombinase enzyme mediated methods that include the binding of a recombinase enzyme to a sequence specific DNA molecule and the subsequent exchange of a linked DNA sequence with a compatible DNA molecule.

"Spacer sequence". The nucleotide sequence located internal to recombinase recognition/inverted repeat sequences of a site-specific recombination system.

Probes and Primers. Nucleic acid probes and primers can be prepared based on a native nucleic acid sequence. A "probe" is an isolated nucleic acid that is attached a conventional detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme. "Primers" are isolated nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods.

Probes and primers are generally 15 nucleotides or more in length, preferably 20 nucleotides or more, more preferably 25 nucleotides, and most preferably 30 nucleotides or more. Such probes and primers preferably hybridize specifically to a target nucleic acid sequence under high stringency hybridization conditions, although for identifying genes that encode homologs of a particular RNA-binding protein such probes or primers may hybridize to a native sequence of another species under moderately stringent conditions.

Methods for preparing and using probes and primers are described, for example, in Sambrook et al., 1989; Ausubel et al., 1992; and Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR-primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

DETAILED DESCRIPTION OF THE INVENTION

Herein we describe and exemplify compositions and methods for modulating recombination for use in the construction of transgene vectors for expression in plants. Genes of interest (GOI) conferring tolerance to an herbicide or antibiotic, insecticidal protein gene, disease resistance genes, genes that affect plant growth, metabolism or development, and genes encoding pharmaceutical proteins, for example, are considered as aspects of the present invention. Such compositions and methods disclosed herein may be used with respect to any plant that can be genetically modified by the methods of plant biotechnology. The compositions and methods herein describe a cloning method for adding segments of DNA to a vector using one or more modified recombination DNA molecules. The methods provides an efficient and novel strategy for the engineering of large fragments of DNA into a vector using successive rounds of a recombination reaction. The method of the present invention, thus provides a novel alternative to conventional cloning methods.

Any site-specific recombination system can be used in accordance with the present invention. More preferably, any similar recombination system wherein the recognition site for the recombinase consists of binding sequences flanked by an asymmetric spacer sequence can be modified and used in a similar manner for adding segments of DNA to a cloning vector. Particularly preferred site-specific recombination systems would include but are not limited to the Cre/lox and FLP/FRT site-specific recombination systems. Both the Cre recombinase derived from bacteriophage P1 and the FLP recombinase derived from *Saccharomyces cerevisiae* mediate site-specific recombination between a pair of target sequences, and are members of the integrase family of recombinases. The chemical structures of over 100 members of the *Int* family of site-specific recombinases have been compared (Nunes-Duby et al., Nucl. Acids Res. 26:391-406, 1998). The recombination mechanism of these systems has also been investigated (Craig, Annu. Rev. Genet. 22:77-105, 1988; Grindley, Curr. Biol., 7:608-612, 1997).

The *loxP* recombination site refers to the target sequence for the Cre recombinase. The *loxP* target sequence comprises two 13-base -pair (bp) inverted repeats, and a central or core 8-bp sequence referred to as the "spacer region". The Cre recombinase catalyzes a reversible reaction, wherein segments of DNA between the wild-type *loxP* sequences can be excised, integrated, or exchanged in a crossover event with another DNA molecule containing a pair of

compatible *loxP* sequences. By compatible as used herein is meant a recombination DNA sequence that is capable of recombination with another recombination DNA sequence by a recombinase-mediated reaction. Accordingly, two wild-type *loxP* sequences are compatible (capable of recombination with each other). Compatible *lox* sequences can also excise DNA as well as integrate DNA. In this regard, compatible *lox* sequences can be useful for cloning methods in which excision of DNA fragments is desired.

The modified *lox* sequences of the present invention are useful for a cloning method requiring addition or integration of segments of DNA. In this regard, mutations in the *loxP* sequence can be made such that the modified sequences are still capable of self-recombination and recombination with other modified sequences, but the recombination product is no longer capable or is significantly reduced or resistant to further recombination. Other modifications can be made that render the *loxP* sequences not capable of recombination with different modified *loxP* sequences. A large number of modified sequences can be made and the recombination site modifications can be classified using a number of testing methods including but not limited to an *in vitro* or *in vivo* analysis of their recombination ability by testing a pair of *lox* sequences, either both on a single plasmid or each *lox* sequence on different plasmids. In one reaction, for example a plasmid DNA molecule containing a wild-type *loxP* can be mixed with a second plasmid containing a modified *lox* sequence in the presence of Cre recombinase. In another reaction, the plasmid DNA molecule containing a modified *lox* site in the presence of Cre can be incubated with another plasmid DNA molecule containing a different modified *lox* site. After the reaction is complete DNA samples can be analyzed on an agarose gel with the appropriate control reactions to evaluate whether or not the plasmids recombined based on the relative intensities of the bands on the gel. For an *in vivo* recombination test, the test plasmids can be transformed in a host organism that expresses the Cre enzyme, and the DNA subsequently isolated and analyzed. In this manner, any number of mutations can be tested, to classify pairs of modified *lox* sequences that are capable of recombination that can then be incorporated in a vector for cloning.

Incompatible modified sequences as used herein refers to modified recombination DNA molecules that are capable of recombination with other modified DNA molecules in the same class, but not with modified DNA molecules in a different class.

Modified *loxP* sequences in the same class are capable of recombining with members of the same class, with varying degrees of efficiency. For example as shown in Table 2, *loxP*02 and *loxP*05 are capable of recombination at a greater efficiency than *loxP*02 and *loxP*04 based on an *in vitro* analysis of recombination. The product of a recombination between two modified recombination sequences such as *loxP*02 and *loxP*05 results in the generation of a new sequence that is incapable, significantly reduced, or resistant to further recombination ability is herein referred to as a "recombination resistant" or "dead" recombination site as shown in Table 3.

In a preferred embodiment, to engineer vectors for the novel cloning method of the present invention one or more pairs of recombination sequences modified within a class can be selected that recombine exclusively with each other, but that then create a "dead" site after the recombination reaction.

In a preferred embodiment, at least one nucleotide of the *loxP* recombination sequence is modified to create mutated *lox* recognition sequences with altered recombination ability. Preferably one or more nucleotides in locations 1 or 8 in the spacer region. More preferably one or more nucleotides in locations 1 or 8 in the spacer region and at least one additional modified nucleotide at positions 2, 5, and 7. Even more preferably modification of one or more nucleotides in positions 1 or 8 of the spacer region and one or more nucleotides of the closest 3 nucleotides to the spacer region in the inverted repeat. By creating select mutations in the *loxP* recombination site, a large number of modified recombination DNA molecules can be used in a novel cloning strategy that takes advantage of "pairing" classes of modified DNAs that are still capable of recombining, but after the recombination are less active to recombinase activity and recombination resistant.

In a preferred embodiment, *lox* sequence mutations in the same class are selected such that the mutated nucleotides are on opposite ends of the 8 base pair spacer region. Consequently, in one example, a cloning strategy can be devised using two *lox* sequences on one vector coupled with two incompatible *lox* sequences on a second vector. More preferably, for construction of a T-DNA double border vector for plant transformation, a half-vector ("two into one") approach involving a donor and recipient plasmid is used (Figure 4). The two *lox* sequences on the first plasmid are selected so that they do not recombine, but have a corresponding *lox* sequence of the same class on the second plasmid that can be used to insert the second plasmid by a double recombination event. The half-vectors are combined in such a

way that the resulting product of recombination is a plasmid that contains all the components of a plant transformation vector including but not limited to a selectable marker, a right border, two multi-cloning sequences in tandem, a left border, replication functions, an inactive recombination site, and an active recombination site. The half-vector approach provides smaller plasmids than conventional binary vectors, and only half of the genes of the final construct need to be cloned by conventional cloning that decreases the need for restriction sites to clone large fragments and decreases the size and number of insert fragments to manipulate. In this method the donor and recipient plasmid can be recombined once, and additional genes can be added to the plasmid by conventional cloning methods. This single round recombination cloning method can be sufficient depending on the desired number of genes to be cloned in the vector.

In another embodiment, a cloning strategy employing at least four modified recombination DNA molecules from two different classes and another recombination DNA molecule from a third class that can be wild type or a modified DNA sequence is suitable for engineering large vectors with multiple genes. For example, in this approach, one plasmid containing a selectable marker, a gene of interest (GOI) and two modified *lox* sequences, plus a third sequence that can be modified or wild type, and a second plasmid containing a different selectable marker and second gene of interest and two modified *lox* sequences, and a third *lox* sequence that can be wild type or modified. Two of these *lox* sequences are capable of recombining with a corresponding *lox* sequence of the same class on the first plasmid, wherein one pair results in a "dead" site. After recombination, a plasmid comprising one selectable marker, two genes of interest, and two functional modified *lox* sequences, at least one sequence remaining for introduction of a second DNA segment with compatible *lox* sequences. Accordingly, multiple rounds of recombination to add additional segments of DNA can be accomplished using this approach. The appropriate multiple "pairs" of *lox* sequences must be selected and vectors engineered such that each addition of a new segment of DNA results in an inactivated site, yet still leaves at least one active recombination site for the next round of recombination. The addition of a third selection device in this method (For example, *sacB* (Quandt et al., Gene, 127:15-21, 1993) can be used to eliminate incomplete products of recombination.

In another embodiment of the invention, a cloning strategy employing multiple site-specific recombination systems can be constructed. For example, combinations of the FLP/FRT

site-specific recombination system for which the recombination site remains capable for recombination and the modified *lox* sequences of the Cre/lox site-specific recombination systems as described in the previous embodiment can be combined to provide a cloning system that utilizes the specific recombinase activities of each system to construct vectors that have expression cassettes stacked to provide multiple phenotypic traits.

Vectors greater than 10 kb can be constructed using the afore described gene-stacking approach without the use of rare cutting restriction enzymes and time intensive screening and purification steps by using appropriate combinations of modified *lox* sequences. In addition, the creation of "dead" sequences eliminates the need to have numerous modified recombination sequences for a particular cloning strategy. The same modified *lox* sequences can be recycled in subsequent cloning steps.

Any mutation or nucleotide change can be made in the recombination site sequence comprising the spacer and inverted repeat regions and result in modified recombination sequences with altered recombination potential. Albert et al., (Plant J., 7:649-659, 1995) disclose that mutations in the inverted repeat can affect the efficiency of recombination. The recombination DNA modifications of the present invention comprise sequences with at least one modification in the 34 base pair region comprising the 8 base pair spacer region and two 13 base pair inverted repeat regions. In this regard, at least one nucleotide change in the recombinase binding site (inverted repeat regions) can be combined with at least one nucleotide change in the spacer region to create modified DNA molecules with varying degrees of recombination efficiency and specificity.

In a preferred embodiment, nucleotide sequences in the spacer region and the 3 nucleotides closest to the spacer region in the inverter repeat sequence are target nucleotide positions for modification of a wild-type recombination site sequence. Those skilled in the art are aware of the methods to modify a nucleotide sequence. One method of alteration of a nucleic acid sequence is to use PCR to modify selected nucleotides or regions of sequences. These methods are known to those of skill in the art. Sequences can be modified, for example by insertion, deletion or replacement of template sequences in a PCR-based DNA modification approach. Modified DNA sequences can be produced, for example, by standard DNA mutagenesis techniques or by chemical synthesis techniques. Methods for cloning and PCR are described, for example, in Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed.

Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989 (hereinafter, "Sambrook et al., 1989"); Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, "Ausubel et al., 1992); and Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR-primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

In a preferred embodiment, nucleic acid sequences can be produced by techniques such as by directly synthesizing the fragment by chemical means, as is commonly practiced by using an automated oligonucleotide synthesizer. Any synthetic DNA fragment including but not limited to wild-type or modified recombination sequences can be designed by inspection of the sequences by the researcher and ordered, for example, through a manufacturer specializing the chemical synthesis of nucleic acids. Alternatively, possible mutations of a recombination site can also be designed by software designed for such purposes.

The method of the present invention can be used with any vector. Those of skill in the art are aware of the numerous types of cloning vectors that can be used for recombinant DNA techniques. Examples of suitable cloning vectors would include but are not limited to plasmids, bacteriophages, cosmids, viral vectors and phagemid vectors for use in any prokaryotic or eukaryotic cloning system.

In a preferred embodiment, the vector comprises one or more non-crossreacting modified recombination sequences as illustrated in Figures 5, 8, and 10-15 and is a plant vector capable of introducing nucleic acids into plants. Any suitable plant vector can be used depending on the plant transformation method. Those of skill in the art are aware of the types of plant expression vectors that can be used in a plant transformation method. A typical plant expression vector for *Agrobacterium*-mediated plant transformation, for example, can include a number of genetic components, including but not limited to a promoter, one or more genes of interest, and a transcription terminator sequence. By genetic component as used herein is meant any nucleic acid sequence or genetic element that may also be a component or part of a vector. The plant expression vector also can contain the functions for mobilization from *E. coli* to *Agrobacterium* and for replication of the vector in these hosts (*i.e. E. coli* and broad host range origin of replication). In addition one or more selectable marker gene(s) for selection of bacterial cells

containing the vector and for selecting plant cells containing the introduced DNA can be components of the plant expression vector. The vector also typically can contain one or more T-DNA borders that function to transfer the DNA to the plant cell. A number of vectors suitable for stable transformation of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., Cloning Vectors: A Laboratory Manual, 1985, supp. 1987); Weissbach and Weissbach, Methods for Plant Molecular Biology, Academic Press, 1989; Gelvin et al., Plant Molecular Biology Manual, Kluwer Academic Publishers, 1990; and R.R.D. Croy Plant Molecular Biology LabFax, BIOS Scientific Publishers, 1993.

Any plant promoter can be used as a 5' regulatory sequence for modulation expression of a particular gene or genes. The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA , and to initiate the transcription into mRNA using one of the DNA strands as a template to make a corresponding complementary strand of RNA. Those of skill in the art are aware of the numerous types of promoters that can be used in a plant expression vector and a number of promoters that are active in plant cells have been described in the literature. A number of promoters have utility for plant gene expression for any gene of interest including but not limited to selectable markers, scorable markers, genes for pest tolerance, disease tolerance, nutritional enhancements and any other gene of agronomic interest. Examples of constitutive promoters useful for plant gene expression include but are not limited to, the cauliflower mosaic virus (CaMV) 35S promoter, that confers constitutive, high-level expression in most plant tissues (see, e.g., Odel et al., Nature 313:810, 1985), including monocots (see, e.g., Dekeyser et al., Plant Cell 2:591, 1990; Terada and Shimamoto, Mol. Gen. Genet. 220:389, 1990); the nopaline synthase promoter (An et al., Plant Physiol. 88:547, 1988) and the octopine synthase promoter (Fromm et al., Plant Cell 1:977, 1989); and the figwort mosaic virus (FMV) promoter. Other types promoters are also envisioned to have utility in the present invention including but not limited to tissue-enhanced promoters, developmentally regulated promoters, or inducible promoters.

Plant expression vectors can include RNA processing signals, e.g., introns, that may be positioned upstream or downstream of a polypeptide-encoding sequence in the transgene. In addition, the expression vectors may include additional regulatory sequences from the 3'-untranslated region of plant genes (Thornburg et al., Proc. Natl. Acad. Sci. USA 84:744 (1987); An et al., Plant Cell 1:115 (1989), e.g., a 3' terminator region to increase mRNA stability of the

mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase 3' terminator regions. 5' non-translated regions of a mRNA can play an important role in translation initiation and can also be a genetic component in a plant expression vector. For example, non-translated 5' leader sequences derived from heat shock protein genes have been demonstrated to enhance gene expression in plants (see, for example U. S. Patent 5,362,865). These additional upstream and downstream regulatory sequences may be derived from a source that is native or heterologous with respect to the other elements present on the expression vector.

The term regulatory sequence as used herein refers to any nucleotide sequence located upstream, within, or downstream to a DNA sequence that controls, mediates, or affects expression of a gene product in conjunction with the protein synthetic apparatus of the cell.

In a preferred embodiment, a genetic component produces a product that serves as a selection device and functions in a regenerable plant tissue to produce a compound that would confer upon the plant tissue resistance to an otherwise toxic compound. Genes of interest for use as a selectable, screenable, or scorable marker would include but are not limited to GUS (coding sequence for beta-glucuronidase), GFP (coding sequence for green fluorescent protein), LUX (coding gene for luciferase), antibiotic resistance marker genes, or herbicide tolerance genes. Examples of transposons and associated antibiotic resistance genes include the transposons Tns (bla), Tn5 (nptII), Tn7 (dhfr), penicillins, kanamycin (and neomycin, G418, bleomycin); methotrexate (and trimethoprim); chloramphenicol; kanamycin and tetracycline.

Characteristics useful for selectable markers in plants have been outlined in a report on the use of microorganisms (Advisory Committee on Novel Foods and Processes, July 1994). These include stringent selection with minimum number of nontransformed tissues, large numbers of independent transformation events with no significant interference with the regeneration, application to a large number of species, and availability of an assay to score the tissues for presence of the marker.

The method of the present invention can be used with any suitable plant transformation plasmid or vector containing one or more non-crossreacting modified recombination sequences, a selectable or screenable marker and associated regulatory elements, as described, along with one or more nucleic acids and associated regulatory elements such that the nucleic acid sequences are expressed in a manner sufficient to confer a particular desirable trait. Examples of suitable DNA coding sequences of agronomic interest envisioned by the present invention would

include but are not limited to one or more genes for insect tolerance, such as *Bacillus thuringiensis* insecticidal proteins, disease resistance including genes for fungal disease control, viral disease control and bacterial disease control, herbicide tolerance such as genes conferring glyphosate tolerance, and genes for quality improvements such as yield, nutritional enhancements, environmental or stress tolerances, or any desirable changes in plant physiology, growth, development, morphology or plant product(s).

Alternatively, the DNA coding sequences can effect these phenotypes by encoding a non-translatable RNA molecule that causes the targeted inhibition of expression of an endogenous gene, for example via antisense- or cosuppression-mediated mechanisms (see, for example, Bird et al., Biotech. Gen. Engin. Rev. 9:207,1991). The RNA could also be a catalytic RNA molecule (i.e., a ribozyme) engineered to cleave a desired endogenous mRNA product (see for example, Gibson and Shillitoe, Mol. Biotech. 7:125,1997). Thus, any gene that produces a protein or mRNA that expresses a phenotype or morphology change of interest are useful for the practice of the present invention.

Any nucleic acid can be introduced into a host cell by the methods encompassed by the present invention include, for example, DNA sequences or genes from another species, or even genes or sequences that originate with or are present in the same species, but are incorporated into recipient cells by genetic engineering methods rather than classical reproduction or breeding techniques. An introduced segment of DNA can be referred to as exogenous DNA. Exogenous as used herein is intended to refer to any gene or DNA segment that is introduced into a recipient cell, regardless of whether a similar gene may already be present in such a cell. The type of DNA included in the exogenous DNA can include DNA that is already present in the plant cell, DNA from another plant, DNA from a different organism, or a DNA generated externally, such as a DNA sequence containing an antisense message of a gene, or a DNA sequence encoding a synthetic or modified version of a gene.

Those of skill in the art are familiar with the standard resource materials that describe specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of recombinant organisms and the screening and isolation of genes, (see for example Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, 1989; Mailga et al., Methods in Plant Molecular Biology, Cold Spring Harbor Press, 1995; Birren et al., Genome Analysis: volume 1,

Analyzing DNA, (1997), volume 2, Detecting Genes, (1998), volume 3, Cloning Systems, (1999) volume 4, Mapping Genomes, (1999), Cold Spring Harbor, New York).

In a preferred embodiment a plant is selected as a target host for the introduction of a novel plant expression vector comprising one or more non-crossreactive modified recombination sequences. Examples of suitable plant targets would include but are not limited to Acadia, alfalfa, apple, apricot, Arabidopsis, artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, castorbean, cauliflower, celery, cherry, chicory, cilantro, citrus, clementines, clover, coconut, coffee, corn, cotton, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, linseed, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, olive, onion, orange, an ornamental plant, palm, papaya, parsley, parsnip, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radiscchio, radish, rapeseed, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, a vine, watermelon, wheat, yams, and zucchini. Particularly preferred monocotyledonous hosts would include cereals such as corn, oats, barley, rice, and wheat. Particularly preferred dicotyledonous hosts would include cotton, potato, and soybean.

The method of the present invention can be used for cloning nucleic acids into vectors useful in any prokaryotic or eukaryotic expression system. In a preferred embodiment, the cloning method is used to construct plant vectors for introducing nucleic acids into plant cells. Accordingly, any plant transformation method can be used. Several methods are available for introducing DNA sequences into plant cells and are well known in the art. Suitable methods include but are not limited to bacterial infection, binary bacterial artificial chromosome vectors, direct delivery of DNA (e.g. via PEG-mediated transformation, desiccation/inhibition-mediated DNA uptake, electroporation, agitation with silicon carbide fibers, and acceleration of DNA coated particles(reviewed in Potrykus, Ann. Rev. Plant Physiol. Plant Mol. Biol., 42: 205, 1991).

Methods for specifically transforming dicots primarily use *Agrobacterium tumefaciens*. For example, transgenic plants reported include but are not limited to cotton (U. S. Patent No. 5,004,863; U. S. Patent No. 5,159,135; U. S. Patent No. 5,518,908, WO 97/43430), soybean (U.

S. Patent No. 5,569,834; U. S. Patent No. 5,416,011; McCabe et al., Bio/Technology, 6:923, 1988; Christou et al., Plant Physiol., 87:671, 1988); *Brassica* (U. S. Patent No. 5,463,174), and peanut (Cheng et al., Plant Cell Rep., 15: 653, 1996).

Similar methods have been reported for the transformation of monocots. Transformation and plant regeneration using these methods have been described for a number of crops including but not limited to asparagus (*Asparagus officinalis*; Bytebier et al., Proc. Natl. Acad. Sci. U.S.A., 84: 5345, 1987); barley (*Hordeum vulgarae*; Wan and Lemaux, Plant Physiol., 104: 37, 1994); maize (*Zea mays*; Rhodes, C.A., et al., Science, 240: 204, 1988; Gordon-Kamm, et al., Plant Cell, 2: 603, 1990; Fromm, et al., Bio/Technology, 8: 833, 1990; Koziel, et al., Bio/Technology, 11: 194, 1993); oats (*Avena sativa*; Somers, et al., Bio/Technology, 10: 1589, 1992); orchardgrass (*Dactylis glomerata*; Horn, et al., Plant Cell Rep., 7: 469, 1988); rice (*Oryza sativa*, including indica and japonica varieties, Toriyama, et al., Bio/Technology, 6: 10, 1988; Zhang, et al., Plant Cell Rep., 7: 379, 1988; Luo and Wu, Plant Mol. Biol. Rep., 6: 165, 1988; Zhang and Wu, Theor. Appl. Genet., 76: 835, 1988; Christou, et al., Bio/Technology, 9: 957, 1991); sorghum (*Sorghum bicolor*; Casas, A.M., et al., Proc. Natl. Acad. Sci. U.S.A., 90: 11212, 1993); sugar cane (*Saccharum* spp.; Bower and Birch, Plant J., 2: 409, 1992); tall fescue (*Festuca arundinacea*; Wang, Z.Y. et al., Bio/Technology, 10: 691, 1992); turfgrass (*Agrostis palustris*; Zhong et al., Plant Cell Rep., 13: 1, 1993); wheat (*Triticum aestivum*; Vasil et al., Bio/Technology, 10: 667, 1992; Weeks T., et al., Plant Physiol., 102: 1077, 1993; Becker, et al., Plant, J. 5: 299, 1994), and alfalfa (Masoud, S.A., et al., Transgen. Res., 5: 313, 1996). It is apparent to those of skill in the art that a number of transformation methodologies can be used and modified for production of stable transgenic plants from any number of target crops of interest.

Those of skill in the art are aware that for *Agrobacterium*-mediated plant transformation methods a number of *Agrobacterium* strains and methods are available. Such strains would include but are not limited to *Agrobacterium* strains C58, LBA4404, EHA101 and EHA105. Particularly preferred strains are *Agrobacterium tumefaciens* strains.

The transformed plants are analyzed for the presence and characteristic(s) conferred by the genes delivered to the plant cells. Those of skill in the art are aware of the numerous methods available for the analysis of transformed plants. A variety of methods are used to assess gene expression and determine if the introduced gene(s) is integrated, functioning

properly, and inherited as expected including but not limited to molecular methods, immunodiagnostic methods, biochemical methods, and field evaluations.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention, therefore all matter set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

EXAMPLES

EXAMPLE 1

Generation of modified *loxP* sequences

The first and the last nucleotides of the spacer region and those nucleotides closest to the spacer nucleotides in the inverted repeats of *loxP* were targeted for nucleotide modifications. Eighteen modified *loxP* sequences were designed, generated and tested. The wild-type *loxP* and modified *loxP* sequences generated are listed in SEQ ID NO:1 and SEQ ID NOS: 5-22 respectively, and a sequence comparison of the *loxP* sequences with the wild-type *loxP* is shown in Table 1. The modified *lox* sequences were generated by annealing corresponding synthetic oligonucleotides ordered from BRL (Life Technologies, Inc., Rockville, MD) with SalI (TCGAC) and PstI (CGAT) restriction endonuclease site overhang sequence added to the 5' and 3' ends, respectively and cloning into SalI, PstI sites of Bluescript SK (-) (available from Life Technologies, Inc. Rockville, MD).

Table 1. Modified *loxP* sequences

*lox*P	ATAACTTCGTATA	ATGTATGC	TATACGAAGTTAT
P01	ATAACTTCGTATA	ATGTATGg	TATACGAAGTTAT
P02	ATAACTTCGTATA	ATGTATGa	TATACGAAGTTAT
P03	ATAACTTCGTATA	ATGTATGt	TATACGAAGTTAT
P04	ATAACTTCGTATA	tTGTATGC	TATACGAAGTTAT
P05	ATAACTTCGTATA	gTGTATGC	TATACGAAGTTAT
P06	ATAACTTCGTATA	cTGTATGC	TATACGAAGTTAT
P07	ATAACTTCGTATA	ATGTATGC	cATACGAAGTTAT

P08	ATAACTTCGTATA	ATGTATGC	TgTACGAAGTTAT
P09	ATAACTTCGTATA	ATGTATGC	TAcACGAAGTTAT
P10	ATAACTTCGTATA	ATGTATGC	cgTACGAAGTTAT
P11	ATAACTTCGTATA	ATGTATGC	TgcACGAAGTTAT
P12	ATAACTTCGTATA	ATGTATGC	cgcACGAAGTTAT
P13	ATAACTTCGTATg	ATGTATGC	TATACGAAGTTAT
P14	ATAACTTCGTAcA	ATGTATGC	TATACGAAGTTAT
P15	ATAACTTCGTgTA	ATGTATGC	TATACGAAGTTAT
P16	ATAACTTCGTAcg	ATGTATGC	TATACGAAGTTAT
P17	ATAACTTCGTgcA	ATGTATGC	TATACGAAGTTAT
P18	ATAACTTCGTgcg	ATGTATGC	TATACGAAGTTAT

EXAMPLE 2

In vitro Evaluation of Recombination Efficiency

Each modified DNA was cloned into two different plasmids of approximately 3.0 kb and 4.0 kb. The larger plasmids were made by cloning a 1.8 kb Hind III-Pst I fragment of pMON21659 (Figure 1) into the corresponding sites of the BlueScript® plasmid (available from a number of manufacturer, see for example BRL, Rockville, MD). pMON21649 is a double border plant transformation vector containing the P-FMV promoter (35S promoter from the Figwort Mosiac Virus); a Hsp70 leader from *Petunia*, the transit peptide region of *Arabidopsis thaliana* EPSP synthase gene (At.EPSPS/CTP2); the CP4 gene (coding sequence for AGRTU.EPSP synthase isolated from strain CP4 as described in U. S. Patent No. 5,633,435); the nos 3' (terminator sequence for the nopaline synthase gene); ori-322 and ori-V origins of replication; Spc/Str (coding region for aad, Aminoglycoside adenyl-transferase that confers resistance to spectinomycin and streptomycin). Plasmids containing two modified *lox* sequences were generated by replacing the Sac I - Eco RV fragment of the large plasmid with the Sac I - Dra I fragment, containing another *lox* sequence, from the small plasmid.

To test for in vitro recombination, the approximately 0.1 μg of each plasmid was mixed (1:1) and the recombination reaction was as described in Abremski and Hoess (J. Biol.Chem, 259:1509-1514, 1984). The 30μl reaction contained 50 mM Tris-HCl, pH 7.5, 33 mM NaCl, 5 mM spermidine, 500 μg/ml BSA, 0.2 μg plasmid DNA and 1 unit of Cre recombinase (Novagen, Madison, WI). The reactions were incubated at 37°C for 30 minutes and stopped by heating at 70°C for 10 minutes. The samples were chilled, supplemented with 10 mM MgCl2, 5 mM DTT and 1 unit of Sst I. The mixture was incubated for 30 minutes at 37°C. Restriction

fragments were separated by agarose gel electrophoresis using a 1% agarose gel using standard methods known to those of skill in the art. The efficiency of recombination in vitro was estimated by visual inspection of the DNA bands on the gel based on the relative amount of the recombined plasmid compared to the relative amount after recombination of the plasmids with two wild-type loxP sequences (loxP x loxP). The results are shown in Table 2.

Table 2. *In vitro* analysis of recombination activity of the modified *loxP* sequences. *lox*P x *lox*P = 100

	loxP	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10	P11	P12	P13	P14	P15	P16	P17	P18
loxP	100																		
P01	75	100																	
P02	25	25	25																
P03	100	100	75	100															
P04	25	25	10	50	0														
P05	100	100	75	100	25	100													
P06	50	50	10	50	0	50	50												
P07	75	75	25	100	10	75	25	100											
P08	50	50	10	50	0	50	25	50	50										
P09	75	100	25	100	10	75	25	100	50	100									
P10	25	50	25	50	0	50	0	0	0	10	0								
P11	25	50	25	50	0	50	0	0	0	10	0	0							
P12	0	50	0	50	0	50	0	0	0	0	0	0	0						
P13	50	50	50	50	0	50	0	50	10	25	25	10	0	50					
P14	75	100	75	100	25	100	10	75	10	75	75	75	0	100	100				
P15	75	100	75	100	50	100	10	75	0	100	75	75	0	100	100	100			
P16	25	50	25	75	0	75	0	10	0	10	0	0	0	0	0	10	10		
P17	50	50	50	50	0	50	0	50	0	25	0	0	0	25	0	25	0	0	
P18	0	10	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

EXAMPLE 3

In vivo evaluation of combinations of modified DNA sequences

Based on the results of the *in vitro* analysis, selected combinations of the modified *lox* sequences were re-evaluated *in vivo* essentially as described in Buchholz et al., (Nucl. Acids Res. 24:3118-3119, 1996). The efficiency of recombination between the selected pairs of *loxP* modified sequence was evaluated (compared with a *loxP* x *loxP* recombination) as well as the efficiency of the recombination product of the two modified ("dead site") with wild-type *loxP*. The results are shown in Table 3. The first number in the cell represents the efficiency of recombination between the two *lox* modified sequences, and the second number in the same cell represents the efficiency of the reverse reaction.

Table 3. *In vivo* Recombination of *loxP* modified sequences

	P01	P02	P03	P07	P08	P09
P04	100/25	25/0	100/25	100/25	25/0	100/25
P05	100/100	100/0	100/100	100/25	100/100	100/100
P06	100/100	25/0	100/100	100/25	100/25	100/100

Pairs of *loxP* modified sequences separated by a 1 kb DNA spacer region were cloned into a single plasmid that was used to transform *E. coli* strain BM25.8 (CLONTECH, Palo, Alto, CA) that expresses Cre recombinase. Freshly transformed cells were grown in liquid medium overnight and then used for plasmid DNA preparation using a standard plasmid DNA isolation kit (see for example Qiagen, Valencia, CA). The plasmid DNA was digested with SspI and separated on a 1% agarose gel. Recombination efficiency was evaluated by presence of non-recombined plasmid in the DNA sample. A control plasmid carrying two wild-type *loxP* sequences yielded exclusively the recombined product.

Recombination between two sequences on the same plasmid resulted in generation of a new smaller plasmid, containing only one site that was a product of recombination of the original two. To test if these recombined "dead" sequences could recombine with the wild-type *loxP* sequence, the *loxP* sequence was cloned into these plasmids from pAYB200*loxP* (Figure 2) as a AflII-SacI fragment into the corresponding sites. pAYB200*loxP* contains two origins of replication (pUC and M13) and a the coding region for Γ-lactamase for ampicillin selection.

The products of recombination between two *loxP* modified sequences are a double modified and a wild-type *loxP* sequence (Figure 3). To check the efficiency of the reverse reaction (double modified recombined site X wild-type *loxP*), each recombined site (ie double modified) was cloned into another plasmid containing a *loxP* sequence. The P02 and P05 modified were selected for further studies. The P02 and P05 *loxP* modified recombined almost as efficiently as two wild-type sequences, but after recombination, the recombination product was a "dead site" that did not exhibit any detectable recombination activity.

EXAMPLE 4

Novel Cloning Strategies

Two different cloning approaches were developed and tested for assembling large megavectors (>10 kb) for Agrobacterium-mediated plant transformation. In one approach referred to as the "Two in One" approach (Figure 4), Cre recombinase mediates the assembly of a binary vector from two "half-vectors." This approach involves two sets of incompatible modified lox sequences that allow the transfer of the region flanked by two lox sequences from the donor to the recipient vector (Table 4). The transfer generates a new plasmid that contains a functional T-DNA cassette and two selectable markers. The lox sequences used in these vectors are listed in Table 4. The lox511 modified site (Hoess et al. Nucl. Acids Res. 14:2287-2300) is present in the backbone of both "half-vectors." These lox511 sequences recombine with each other and remain active after recombination. The loxP02 modified recombination sequence is present in the T-DNA region of the donor vector and the loxP05 modified recombination sequence is present in the T-DNA of the recipient. These two modified sequences also recombine with each other, but generate a "dead" site in the T-DNA. By dead site as used herein is meant the sequences are significantly less efficient than any remaining sequences. Efficiency of recombination is determined as set forth in Table 2 and Table 3 as described in Example 2 and Example 3.

Table 4. Modified *lox* sequences used in the "Two into One" Cloning Method

ATAACTTCGTATA ATGTATAC TATACGAAGTTAT lox511

ATAACTTCGTATA ATGTATGA TATACGAAGTTAT loxP02

ATAACTTCGTATA GTGTATGC TATACGAAGTTAT loxP05

The donor and recipient vectors used for the "Two into One" unconventional cloning approach are shown in Figure 5. The donor (pMON38260) contains the pUC origin of replication, lox511 site, spectinomycin resistance gene, a right T-DNA border, a multicloning site (MCS), and a *loxP*05 modified sequence. The recipient contains a pUC and V origins of replication, the gentamycin resistance gene, two incompatible modified sequences, lox511 *loxP*02, cloned next to each other, a left T-DNA border and a multicloning site. After the genes of interest were cloned into the multicloning sites of the half-plasmids, plasmid DNAs were mixed 1:1 and incubated with Cre recombinase as described previously. After the recombination, the reaction mix was used directly for *E. coli* transformation. The cells were

plated on LB-agar plates supplemented with spectinomycin (100 μg/ml) and gentamycin (5 μg/ml). The plasmids from the colonies resistant to both antibiotics were analyzed by restriction enzyme analysis to distinguish the desirable double recombination events from single recombination events (Figure 6).

EXAMPLE 5

Gene-Stacking Approach

To enable the sequential addition of new genes to an existing cassette, a second approach employing five *loxP* modified sequences was developed (Figure 7). Five *loxP* modified sequences: lox511, *loxP*02, *loxP*05, loxA02 and loxA05, that represent 3 incompatibility classes of the modified sequences (Table 5). Lox511 recombines only with itself. *LoxP*02 recombines exclusively with *loxP*05. LoxA02 recombines exclusively with loxA05. The last two reactions were irreversible and resulted in the generation of double or triple modified *loxP* sequences.

Table 6 provides an additional set of *lox* modified sequences that corresponds to those listed in Table 1 with exception of a single modification in the spacer region. A *loxP* modified sequence designated loxA has a "C" nucleotide base in place of the "T" in the position 2 of the 8 base pair spacer region. Variants of the novel loxA modified sequences have the loxA modification in addition to the nucleotide change of the corresponding *loxP* modified sequence. These new modified loxA sequences recombine with each other at the same rate as the corresponding *lox*P *sequences* (Table 2, 3), but not with any of *lox*P *sequences*. Similarly, the lox511 *sequence* (Hoess et al., Nucl. Acids Res. 14:2287-2300, 1986) can be modified to create an additional novel combination of the recombination sequences incompatible neither with *lox*P nor loxA *sequences* (Table 7). Similar sets of the *sequences* designated 5171 and 2272 (Lee and Saito, Gene 216:55-65, 1998) made with two nucleotide changes in the spacer region are listed in Table 8 and Table 9 respectively.

Table 5. The *lox* modified sequence used for the 'gene-stacking' unconventional cloning.

ATAACTTCGTATA ATGTATAC TATACGAAGTTAT	lox511
ATAACTTCGTATA ATGTATGA TATACGAAGTTAT	loxP02
ATAACTTCGTATA GTGTATGC TATACGAAGTTAT	loxP05
ATAACTTCGTATA ACGTATGA TATACGAAGTTAT	loxA02
ATAACTTCGTATA GCGTATGC TATACGAAGTTAT	loxA05

Table 6. Combination multiple modified sequence of loxA plus *loxP* nucleotide changes

*lox*A	ATAACTTCGTATA	AcGTATGC	TATACGAAGTTAT
A01	ATAACTTCGTATA	AcGTATGg	TATACGAAGTTAT
A02	ATAACTTCGTATA	AcGTATGa	TATACGAAGTTAT
A03	ATAACTTCGTATA	AcGTATGt	TATACGAAGTTAT
A04	ATAACTTCGTATA	tcGTATGC	TATACGAAGTTAT
A05	ATAACTTCGTATA	gcGTATGC	TATACGAAGTTAT
A06	ATAACTTCGTATA	ccGTATGC	TATACGAAGTTAT
A07	ATAACTTCGTATA	AcGTATGC	cATACGAAGTTAT
A08	ATAACTTCGTATA	AcGTATGC	TgTACGAAGTTAT
A09	ATAACTTCGTATA	AcGTATGC	TAcACGAAGTTAT
A10	ATAACTTCGTATA	AcGTATGC	cgTACGAAGTTAT
A11	ATAACTTCGTATA	AcGTATGC	TgcACGAAGTTAT
A12	ATAACTTCGTATA	AcGTATGC	cgcACGAAGTTAT
A13	ATAACTTCGTATg	AcGTATGC	TATACGAAGTTAT
A14	ATAACTTCGTAcA	AcGTATGC	TATACGAAGTTAT
A15	ATAACTTCGTgTA	AcGTATGC	TATACGAAGTTAT
A16	ATAACTTCGTAcg	AcGTATGC	TATACGAAGTTAT
A17	ATAACTTCGTgcA	AcGTATGC	TATACGAAGTTAT
A18	ATAACTTCGTgcg	AcGTATGC	TATACGAAGTTAT

Table 7. Combination multiple modified sequence of lox511 plus *loxP* nucleotide changes

*lox*511	ATAACTTCGTATA	ATGTATaC	TATACGAAGTTAT
51101	ATAACTTCGTATA	ATGTATag	TATACGAAGTTAT
51102	ATAACTTCGTATA	ATGTATaa	TATACGAAGTTAT
51103	ATAACTTCGTATA	ATGTATat	TATACGAAGTTAT
51104	ATAACTTCGTATA	tTGTATaC	TATACGAAGTTAT
51105	ATAACTTCGTATA	gTGTATaC	TATACGAAGTTAT
51106	ATAACTTCGTATA	cTGTATaC	TATACGAAGTTAT
51107	ATAACTTCGTATA	ATGTATaC	cATACGAAGTTAT
51108	ATAACTTCGTATA	ATGTATaC	TgTACGAAGTTAT
51109	ATAACTTCGTATA	ATGTATaC	TAcACGAAGTTAT
51110	ATAACTTCGTATA	ATGTATaC	cgTACGAAGTTAT
51111	ATAACTTCGTATA	ATGTATaC	TgcACGAAGTTAT
51112	ATAACTTCGTATA	ATGTATaC	cgcACGAAGTTAT
51113	ATAACTTCGTATg	ATGTATaC	TATACGAAGTTAT
51114	ATAACTTCGTAcA	ATGTATaC	TATACGAAGTTAT
51115	ATAACTTCGTgTA	ATGTATaC	TATACGAAGTTAT
51116	ATAACTTCGTAcg	ATGTATaC	TATACGAAGTTAT
51117	ATAACTTCGTgcA	ATGTATaC	TATACGAAGTTAT
51118	ATAACTTCGTgcg	ATGTATaC	TATACGAAGTTAT

Table 8. Combination multiple modified sequence of lox5171 plus *loxP* nucleotide changes

*lox*5171	ATAACTTCGTATA	ATGTgTaC	TATACGAAGTTAT
517101	ATAACTTCGTATA	ATGTgTag	TATACGAAGTTAT
517102	ATAACTTCGTATA	ATGTgTaa	TATACGAAGTTAT
517103	ATAACTTCGTATA	ATGTgTat	TATACGAAGTTAT
517104	ATAACTTCGTATA	tTGTgTaC	TATACGAAGTTAT
517105	ATAACTTCGTATA	gTGTgTaC	TATACGAAGTTAT
517106	ATAACTTCGTATA	cTGTgTaC	TATACGAAGTTAT
517107	ATAACTTCGTATA	ATGTgTaC	cATACGAAGTTAT
517108	ATAACTTCGTATA	ATGTgTaC	TgTACGAAGTTAT
517109	ATAACTTCGTATA	ATGTgTaC	TAcACGAAGTTAT
517110	ATAACTTCGTATA	ATGTgTaC	cgTACGAAGTTAT
517111	ATAACTTCGTATA	ATGTgTaC	TgcACGAAGTTAT
517112	ATAACTTCGTATA	ATGTgTaC	cgcACGAAGTTAT
517113	ATAACTTCGTATg	ATGTgTaC	TATACGAAGTTAT
517114	ATAACTTCGTAcA	ATGTgTaC	TATACGAAGTTAT
517115	ATAACTTCGTgTA	ATGTgTaC	TATACGAAGTTAT
511716	ATAACTTCGTAcg	ATGTgTaC	TATACGAAGTTAT
517117	ATAACTTCGTgcA	ATGTgTaC	TATACGAAGTTAT
517118	ATAACTTCGTgcg	ATGTgTaC	TATACGAAGTTAT

Table 9. Combination multiple modified sequence of lox2272 plus *loxP* nucleotide changes

*lox*2272	ATAACTTCGTATA	AaGTATcC	TATACGAAGTTAT
227201	ATAACTTCGTATA	AaGTATcg	TATACGAAGTTAT
227202	ATAACTTCGTATA	AaGTATca	TATACGAAGTTAT
227203	ATAACTTCGTATA	AaGTATct	TATACGAAGTTAT
227204	ATAACTTCGTATA	taGTATcC	TATACGAAGTTAT
227205	ATAACTTCGTATA	gaGTATcC	TATACGAAGTTAT
227206	ATAACTTCGTATA	caGTATcC	TATACGAAGTTAT
227207	ATAACTTCGTATA	AaGTATcC	cATACGAAGTTAT
227208	ATAACTTCGTATA	AaGTATcC	TgTACGAAGTTAT
227209	ATAACTTCGTATA	AaGTATcC	TAcACGAAGTTAT
227210	ATAACTTCGTATA	AaGTATcC	cgTACGAAGTTAT
227211	ATAACTTCGTATA	AaGTATcC	TgcACGAAGTTAT
227212	ATAACTTCGTATA	AaGTATcC	cgcACGAAGTTAT
227213	ATAACTTCGTATg	AaGTATcC	TATACGAAGTTAT
227214	ATAACTTCGTAcA	AaGTATcC	TATACGAAGTTAT
227215	ATAACTTCGTgTA	AaGTATcC	TATACGAAGTTAT

227216	ATAACTTCGTAcg	AaGTATcC	TATACGAAGTTAT
227217	ATAACTTCGTgcA	AaGTATcC	TATACGAAGTTAT
227218	ATAACTTCGTgcg	AaGTATcC	TATACGAAGTTAT

A binary vector was constructed along with two different shuttle vectors (Figure 8). The binary vector contained a lox511 modified sequence, positioned between the promoter and the coding region of the spectinomycin resistance gene, a right T-DNA border, a loxP02 modified sequence, a multicloning site, and a left T-DNA border. The plasmid also contained an antibiotic marker to confer resistance to spectinomycin.

Shuttle vector I contained a lox511 site, adjacent to the promoterless coding region of the hygromycin resistance gene, the right T-DNA border, loxA02 modified sequence, a multicloning site, and a *loxP*05 modified sequence. Site-specific recombination of the binary vector with the shuttle I vector resulted in replacement of the region flanked by lox511 and *loxP*02 sequences with the region flanked by lox511 and *loxP*05 sequences from shuttle I. The recombined plasmid lost resistance to spectinomycin, but gained resistance to hygromycin. In addition, the loxA02 modified sequence along with the genes of interest cloned into the shuttle I were introduced downstream from the right T-DNA border of the binary vector, and *loxP*02 sequence was destroyed after recombination with the *loxP*05 sequence in shuttle I.

Shuttle II contained a lox511 modified sequence, adjacent to the promoterless coding region of the spectinomycin resistance gene, the right T-DNA border, *loxP*02 modified sequence, a multicloning site, and loxA05 modified sequence. After recombination of the shuttle II with the product of the first round of recombination, the spectinomycin resistance gene was restored, the *loxP*02 sequence was reintroduced, additional genes of interest were transformed from the shuttle, and the loxA02 sequence was destroyed. After two rounds of recombination, all the functional elements of the binary vector were restored, and the vector could be used again for recombination with the shuttle I, carrying additional genes of interest.

Thus the gene-stacking approach allows the recycling of the same modified *lox* sequence and the two shuttle vectors can be used repeatedly in sequence to add additional genes of interest. In addition to the elements described above, both shuttle vectors contained the *sacB* gene that allows for direct selection for gene replacement (Quandt et al., Gene, 127:15-21, 1993). The products of recombination of the basic vector with shuttle I (incomplete recombination and complete recombination) are shown in Figure 9.

EXAMPLE 6

Construction of pMON38267 and expression analysis in transgenic potato.

The donor vector (pMON38264, Figure 11) contains the pUC origin of replication, lox511 sequence, spectinomycin resistance gene, a right T-DNA border, the genetic elements of the expression cassette for potato leaf roll virus (PLRV) resistance (P-FMV/L-Gm.Hsp17.9/PLRV.rep/T-Ps.RbcS:e9), the expression cassette for glyphosate tolerance (P-FMV/TS-At.EPSPS:CTP2/T-Ps.RbcS:e9) and a *loxP*05 modified sequence. The recipient vector (pMON38254, Figure 10) contains a pUC and ori-V origins of replication, the gentamycin resistance gene, two incompatible modified recombination sequences, lox511 and *loxP*02, cloned next to each other, an expression cassette for potato virus Y (PVY) resistance (P-FMV/LGm.Hsp17.9/PVY cp/T-Ps.RbcS:e9), an expression cassette for colorado potato beetle (CPB) resistance (P-At.RbcS:1A/Bt.CryIIIA/T-nos), and a left T-DNA border. The genetic elements and expression cassettes of the donor and recipient vectors were made by conventional restriction enzyme digests and ligation with T4 DNA ligase.

The vector plasmids were mixed 1:1 and incubated with Cre recombinase as previously described. After the recombination reaction, the reaction mix was used directly for *E. coli* transformation. The transformed cells were plated on LB-agar plates supplemented with spectinomycin (100 μg/ml) and gentamycin (5 μg/ml). The plasmids from the colonies resistant to both antibiotics were isolated by miniprep DNA methods and analyzed by restriction enzyme analysis to distinguish the desirable double recombination events to create pMON38267 (Figure 12).

The pMON38267 vector was mated into *Agrobacterium* ABI by a triparental mating procedure (Ditta et al., Proc Natl Acad Sci USA 77: 7347-7351 (1980). Transformation of potato was performed by an *Agrobacterium*-mediated transfer of genetic material (Tinland, B. Trends in Plant Sci 1: 178-184 (1996), specifically by the method of Newell *et al.*, (Plant Cell Rep 10:30-34 (1991), or a modification of this method using glyphosate as the selectable marker. Independent regenerated shoots were rooted and assigned line identification numbers. A large number of independent lines were regenerated. These were screened by biochemical and bioassay for expression of the gene product or desirable phenotype conferred by the expression cassettes contained in the vector made by the method of this invention. Table 10

illustrates that the expression cassettes are intact and express the product and desirable agronomic phenotype.

The potato lines (*Solanum tuberosum* var Atlantic) were screened by bioassay for PVY resistance by first inoculating the plants with PVY virus, then analysis by enzyme linked immunosorbent assay (ELISA) for the presence of the virus (Kaniewski et al., Bio/Technology 8:750-754). Column 2 of Table 10 shows PVY accumulation determined by PVY-ELISA before inoculation/14 days after inoculation, these lines were highly or moderately resistant to PVY accumulation. The Bt CRYIIIA protein expression was determined by CRYIIIA ELISA (Perlak et al., Plant Mol Biol 22: 313-321 (1993), and expressed as parts per million (ppm), all of these lines produced sufficient CRYIIIA protein to control the beetle pest. The expression of the EPSPS protein expressed from the transgene cassette was determined by ELISA, all of these lines produced the transgene EPSPS protein. The ELISA (enzyme linked immunosorbent assay) procedure used to quantitate CP4 Enol-Pyruvyl-Shikimate-3-Phosphate Synthase (EPSPS) in whole plant tissues. The assay described in this procedure is a direct ELISA that quantitates the levels of CP4 EPSPS protein present in plant tissue extracts. The plant tissue is extracted in 20:1 volume/weight of buffer in a Brinkmann polytron mechanical homogenizer at 17.5 k rpm for 30 seconds. A single centrifugation step at 6,660g for 8 min. separates the insoluble debris from the soluble extract. The levels in plant samples are compared to a purified reference standard of CP4 EPSPS isolated from *Escherichia coli*. In brief, 96-well polystyrene plates are coated with purified goat anti-CP4 (2 µg/well) then blocked with non-fat dry milk (1% in 1XPBST buffer, phosphate buffered saline pH 7.4, 0.05% Tween-20) for 30 min. at 30 C, then washed 3 times with 1XPBST. Two hundred-fifty µl of soluble plant tissue extract/well (the extract may be diluted with 1XPBST as needed) is added to the antibody-coated wells alongside a concentration range of pure CP4 EPSPS protein standards. The plates are incubated allowing antigen capture by the surface bound antibodies. The unbound sample is washed away with buffer and rabbit anti-CP4 EPSPS conjugated to horseradish peroxidase (1:170 in 1XPBST) is added, binding to the CP4 EPSPS antigen. Following incubation and washing, peroxidase substrate is added to each well. Wells containing CP4 EPSPS and hence, the antibody sandwich (goat anti-CP4 EPSPS + plant CP4 EPSPS + rabbit anti-CP4 EPSPS horseradish peroxidase), will turn blue. The peroxidase TMB substrate and hydrogen peroxide buffers (cat# 50-76-02, Kirkegaard & Perry Labs) reaction results in a soluble blue product and when the reaction is stopped with 3M

phosphoric acid, the product turns yellow. Quantitation of sample CP4 EPSPS concentration is accomplished by extrapolation (based on sample absorbance value obtained from and ELISA plate reader, read at 450 nm with a reference wavelength of 655 nm) from the log-log quadratic regression curve fit of the CP4 EPSPS standard curve ranging from (0.1ng - 2.0 ng CP4/well) or (0.4ng- 8.0 ng CP4/ml). Analysis of the PLRV resistance cassette is by bioassay. The potato plants were inoculated with PLRV and percent infection was determined by ELISA (Thomas et al., Mol Breed 4: 407-417 (1998).

Table 10 . Analysis of pMON38267 efficacy in transgenic potato

Line #	PVY-ELISA	CRYIIIA (ppm)	EPSPS:CP4m (ppm)	PLRV infection (%)
At-8 control	0.315/0.619	ND	ND	22.2
At-B-002	0.212/0.278	51.5	5.3	0.0
At-B-015	0.271/0.279	31.5	8.3	0.0
At-B-008	0.239/0.296	18.3	20.3	0.0
At-B-010	0.250/0.322	20.7	102.2	0.0
At-B-003	0.228/0.321	13.2	8.2	0.0

EXAMPLE 7

Construction of pMON54017 and expression analysis in transgenic potato.

The donor vector (pMON38993, Figure 14) contains the pUC origin of replication, lox511 sequence, spectinomycin resistance gene, a right T-DNA border, the genetic elements of the expression cassette for enhanced starch biosynthesis (P-Pat:1.0/TS-At.RbcS:1A/GlgC16/T-AGRTU.nos), the expression cassette for reduced black spot bruising phenotype (P-TFM7/St.asPPO//T-Ps.RbcS:e9) and a *loxP*05 modified sequence. The genetic element components of pMON38993 include the P-Pat:1.0 promoter as described in Bevan et al. Nucleic Acids Research 14:4625-4638 (1986), the chloroplast targeting sequence of the rubisco small subunit 1A gene, the GlgC16 ADPglucose pyrophosphorylase gene as described in by Barry et al., (US Patent No 5,608,149), the nopaline synthase 3' termination region from *Agrobacterium tumefaciens*, the P-TFM7 promoter as described in Santino et al., (Plant.Mol. Biol. 33:405-416 (1997), the potato polyphenol oxidase gene as described in Thygesen et al. (Plant Physiology

109:525-531 (1995), and the 3' termination region from pea rubisco small subunit gene. The recipient vector (pMON38986, Figure 13) contains a pUC and ori-V origins of replication, the gentamycin resistance gene, two incompatible modified recombination sequences, lox511 and *loxP*02, cloned next to each other, an expression cassette for plant tolerance to glyphosate herbicide (P-FMV/L-Ph.Hsp70/TS-At.EPSPS:CTP2/AGRTU.aroA:CP4/T-AGRTU.nos), an expression cassette for enhanced sucrose biosynthesis (P-FMV/Fda//T-AGRTU.nos), and a left T-DNA border. The genetic element components of pMON38986 include the 35S promoter from the Figwort mosaic virus (P-FMV) (US Patent No 5,378,619), the 5' leader sequence of the heat shock 70 gene from *Petunia hybrida* (Ph.Hsp70), the chloroplast targeting signal from the *Arabidopsis thaliana* EPSPS gene (Klee et al., Mol. Gen. Genet. 210:437-442 (1987), the glyphosate resistant EPSPS enzyme from *Agrobacterium tumefaciens* strain CP4 (US Patent No 5,633,435), the nopaline synthase 3' termination region from *Agrobacterium tumefaciens*, and the fructose 1,6 diphosate aldolase gene (WO 98/58069). The genetic elements and expression cassettes of the donor and recipient vectors were assembled by conventional restriction enzyme digests and ligation with T4 DNA ligase. Additional genetic elements known to those skilled in the art of plant genetic engineering can be combined as well as modification of nucleic acid sequence for improved plant expression, for a review of optimizing expression of transgenes in plants, see Koziel et al., (Plant Mol. Biol. 32:393-405 (1996),

The vector plasmids were mixed 1:1 and incubated with Cre recombinase as previously described. After the recombination reaction, the reaction mix was used directly for *E. coli* transformation. The transformed cells were plated on LB-agar plates supplemented with spectinomycin (100 μg/ml) and gentamycin (5 μg/ml). The plasmids from the colonies resistant to both antibiotics were isolated by miniprep DNA methods and analyzed by restriction enzyme analysis to distinguish the desirable double recombination events to create pMON54017 (Figure 15).

The pMON54017 vector was mated into *Agrobacterium* ABI by a triparental mating procedure (Ditta et al., Proc Natl Acad Sci USA 77: 7347-7351 (1980). Transformation of potato was performed by an *Agrobacterium*-mediated transfer of genetic material (Tinland, B. Trends in Plant Sci 1:178-184 (1996), specifically by the method of Newell *et al.*, (Plant Cell Rep 10:30-34 (1991), or a modification of this method using glyphosate as the selectable marker. Independent regenerated shoots were rooted and assigned line identification numbers.

A large number of independent lines were regenerated. These were screened by biochemical and bioassay for expression of the gene product or desirable phenotype conferred by the expression cassettes contained in the vector made by the method of this invention.

Fda ELISA was performed on the regenerated potato lines to determine expression of the Fda gene product. Approximately 25 mg of leaf tissue was collected with a small cork borer from each line and homogenized in 500 µl of phosphate buffered saline with tween-20 (PBST). Microtiter plates were coated with 100 µl/well anti-Fda IgG at 1 mg/ml (1:1000) in antibody coating buffer for 4 hrs at room temperature or overnight at 4 C. The plates were washed and the 100 µl of the leaf homogenate diluted 1:10 in PBST-0.2% bovine serum albumin (BSA) was loaded into the wells of the coated microtiter plate, appropriate negative and positive controls were included. The plates are incubated for 4 hrs at room temperature or overnight at 4 C. The plate is washed and 100 µl anti-Fda alkaline phosphatase conjugate antibody (1:1500) in PBST-0.2% BSA is added to each well. Incubate, wash, then add 100 µl/well alkaline phosphatase substrate buffer (1 mg/ml PNPP in 0.2 M Tris buffer) to each well. Read optical density at 405 nm reference 650 nm after approximately 45 minutes. Lines were scored +/- for the presence of the transgenic Fda protein.

GlgC16 ELISA was performed on tissue extracts according to manufacturer's instructions Cat# 76050 (Strategic Diagnostic Inc. NJ). Lines were scored +/- for the presence of the transgenic GlgC16 protein.

PPO-Oxidative Browning Assay Of Tuber Homogenates. For each transgenic line, measurements were performed on samples of tuber tissue (approximately 1-5 grams) extracted by a #8 cork punch from the center of each tuber. Tuber tissue was homogenized using a polytron on speed five for approximately 30 seconds, in 20 mM sodium acetate buffer, pH 5.2, at a 1:5 (w/v) tissue to buffer ratio. Proteins (usually 5 µl) were analyzed in duplicate by Bradford assay. The homogenate was allowed to oxidize at 22 C for 20 hours in the same round bottom tube, centrifuged at 12,000 for 5 minutes, and decanted for optical density measurements. Optical density was directly measured at 475 nm. Units of oxidative browning rate were calculated as optical density after 20 hours divided by mg of protein on a per ml basis (divided by mg protein/ml). In the case of greenhouse minitubers, tyrosine was optionally added to the homogenates at a final concentration of 2.0 mM in order to intensify the brown color development after 20 hours. The results in column of Table 11 show that by this assay method

no reduction in oxidative browning was detected. The low efficiency of the antisense gene silencing necessitates screening a sufficiently large number of transgenic lines for the desired phenotype in this example a reduction in product from the oxidative assay.

Tissue culture selection of the transgenic potato lines on glyphosate containing media determined that the lines expressed sufficient levels of the resistant EPSPS enzyme to allow root formation and shooting of the plants. Potato lines that shooted on media containing 0.025 mM glyphosate were determined to be positive for gene expression.

Table 11. Analysis of pMON54017 efficacy in transgenic potato

Line #	Fda ELISA	GlgC16 ELISA	Oxidative assay	glyphosate tolerance
S10308	+	+	-	+
S10314	+	+	-	+
S10317	+	+	-	+
S10322	+	+	-	+
S10333	+	+	-	+

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application is specifically and individually indicated to be incorporated by reference.

CLAIMS:

1. DNA vector comprising a first DNA molecule of an integrase family site-specific recombination system comprising a first spacer sequence and a first recombinase recognition/inverted repeat sequence, wherein said first spacer sequence is modified for at least one nucleotide relative to native spacer sequence at position 1 or 8;

said first DNA molecule linked to a DNA segment;

said DNA segment linked to a second DNA molecule of the same integrase family site-specific recombination system as the first DNA molecule comprising a second spacer sequence and a second recombinase recognition/inverted repeat sequence, wherein said second spacer sequence is modified for at least one nucleotide at position 1 or 8, different than said modified nucleotide of the first spacer sequence, wherein said first and second DNA molecules are incompatible for recombination.

2. A DNA vector of claim 1, wherein said first and second spacer sequences are additionally independently modified at at least one of the positions selected from the group 2, 5, and 7.

3. A first or second DNA molecule of claim 1, wherein said site-specific recombination system is Cre/Lox.

4. A first or second DNA molecule of claim 3, wherein said nucleotide is selected from the group consisting of A, G, C, and T.

5. A DNA vector of claim 1, 2, 3 or 4, wherein said first or second DNA segments comprise genetic elements that function in plants.

6. A DNA vector of claim 5, wherein said genetic elements comprise promoters, introns, leaders, coding sequences, antisense sequences, and 3' terminators.

7. A DNA vector of claim 5, wherein said first or second DNA segments comprise expression cassettes that function in plants, wherein said expression cassettes have genetic elements operably linked to express a product in plants.

8. A DNA vector of claim 4, wherein said first or second DNA segments comprise polylinker sequence.

9. A DNA vector comprising a first DNA molecule of an integrase family site-specific recombination system consisting essentially of a first spacer sequence and a first recombinase recognition/inverted repeat sequence, wherein said first spacer sequence is modified relative to native sequence at any nucleotide selected from the group consisting of position 2, 5, 7, and said

first recombinase recognition/inverted repeat sequence is modified relative to native sequence for at least one nucleotide in any of the three nucleotides flanking the ends of the first spacer sequence;

said first DNA molecule linked to a DNA segment;

said DNA segment linked to a second DNA molecule of the same integrase family site-specific recombination system as the first DNA molecule consisting essentially of a second spacer sequence and a second recombinase recognition/inverted repeat sequence, wherein said second spacer sequence is modified relative to native sequence at any nucleotide selected from the group consisting of position 2, 5, 7, and said second recombinase recognition/inverted repeat sequence is modified relative to native sequence for at least one nucleotide in any of the three nucleotides flanking the ends of the first spacer sequence; wherein said first and second DNA molecules are incompatible for recombination.

10. A DNA vector or claim 9, wherein said first and second spacer sequences are the native sequences.

11. A first or second DNA molecule of claim 9 wherein said site-specific recombination system is Cre/Lox.

12. A DNA molecule of claim 11 wherein said nucleotide is selected from the group consisting of A, G, C, and T.

13. A DNA molecule of claim 12 wherein said modification of said first and second recombinase recognition/inverted repeat sequence occurs to any one of the three nucleotides flanking the ends of the spacer sequence.

14. A DNA molecule of claim 12 wherein said modification of said first and second recombinase recognition/inverted repeat sequence occurs to any two of the three nucleotides flanking the ends of the spacer sequence.

15. A DNA molecule of claim 12 wherein said modification of said first and second recombinase recognition/inverted repeat sequence occurs to any three of the three nucleotides flanking the ends of the spacer sequence.

16. A DNA molecule of a site-specific recombination system of claims 4, 13, 14, or 15, selected from the group consisting of SEQ ID NO:5-97.

17. A method for adding a DNA segment into a vector comprising a site-specific DNA recombination system comprising the steps of:

A) selecting modified recombination DNA molecules of claim 16;

B) constructing a recipient vector to contain a first modified recombination DNA molecule linked to a first DNA segment, said first DNA segment linked to a second modified recombination DNA molecule that is incompatible for recombination with said first modified recombination DNA molecule;

C) constructing a donor vector to contain a third modified recombination DNA molecule that is recombination compatible with said first recombination DNA molecule and linked to a second DNA segment, wherein upon recombination with the recipient vector is recombination resistant, said second DNA segment linked to a fourth modified recombination DNA molecule that is recombination incompatible with said third recombination DNA molecule and recombination compatible to said second recombination DNA molecule, wherein upon recombination with the recipient vector results in a first combined modified recombinant DNA molecule that is recombination reaction resistant and a second combined modified recombinant DNA molecule that is recombination reaction susceptible;

D) combining recipient vector and donor vector in the presence of a recombinase enzyme capable of catalyzing DNA recombination between the DNA molecules to produce a recombinant DNA product.

18. A method of claim 15 wherein said second combined modified recombinant DNA molecule is recombination reaction resistant.

19. A method of claim 17 wherein said first or second DNA segments comprise genetic elements that function in plants.

20. A method of claim 19 wherein said genetic elements comprise promoters, introns, leaders, coding sequences, antisense sequences, and 3' terminators.

21. A method of claim 19 wherein said first or second DNA segments comprise expression cassettes that function in plants, wherein said expression cassettes have genetic elements operably linked to express a product in plants.

22. A method of claim 17 wherein said first or second DNA segments comprise polylinker sequence.

23. A method for the sequential stacking of DNA segments into a vector comprising

A) selecting a plurality of recombination DNA molecules of claim 16;

B) arranging pairs of recombination incompatible DNA molecules linked to intervening DNA segments in a series in a first donor vector;

C) constructing a first recipient vector containing at least two recombination DNA molecules compatible with at least two DNA recombination molecules of the first donor vector and a third recombination DNA molecule incompatible with any of the recombination DNA molecules of the donor and recipient vector;

D) recombining of said DNA segments in the presence of a recombinase enzyme capable of catalyzing DNA recombination between the compatible DNA molecules in a first recombination reaction, wherein upon recombination at least one of the recombinant DNA molecules is resistant to further recombination reaction;

E) identifying recombinate DNA segments;

F) repeating the sequential process of adding DNA segments by recombination reaction to said recipient vector from a third donor vector comprising a fourth and fifth recombination DNA molecule, said fourth recombination DNA molecule compatible with said third recombination DNA molecule and said fifth recombination DNA molecule compatible with any one of the recombination DNA molecules resulting from the first recombination reaction;

G) repeating step D and step E;

H) repeating steps F, then D, then E to add DNA segments by selection of flanking compatible and incompatible DNA molecules and recombination with compatible recombination DNA molecules of the vector resulting from the previous step;

I) repeating step H until as many DNA segments are added as desirable.

24. A method of claim 23, wherein said DNA segments comprise genetic elements that function in plants.

25. A method of claim 24, wherein said genetic elements are selected from the group consisting of promoters, introns, leaders, coding sequences, antisense sequences, and 3' terminators.

26. A method of claim 23 wherein said DNA segments comprise expression cassettes, wherein said expression cassettes function in plants and have genetic elements operably linked to express a product in plants

27. A method of claim 23 wherein said DNA segments comprise polylinker sequence.

28. A transgenic plant cell comprising a recombinant DNA molecule of a site-specific recombination system resistant to further recombinase activity and a recombinant DNA molecule of the same site-specific recombination system susceptible to further recombinase activity wherein the DNA molecules reside in the genome of the plant cell.

29. A transgenic plant cell comprising a recombinant DNA molecule of a site-specific recombination system resistant to further recombinase activity

30. A transgenic crop plant comprising a recombinant DNA molecule of a site-specific recombination system resistant to further recombinase activity and a recombinant DNA molecule of the same site-specific recombination system susceptible to further recombinase activity wherein the DNA molecules reside in the genome of the crop plant.

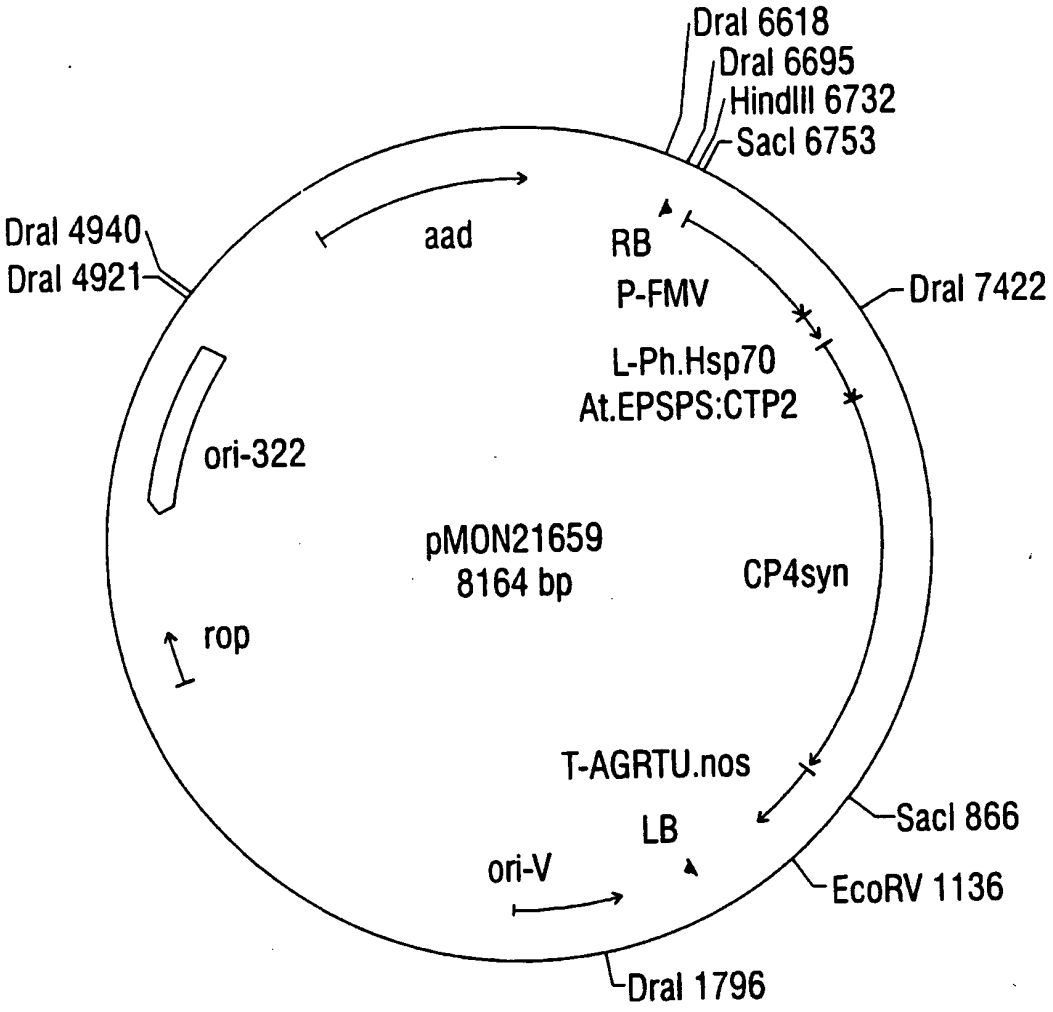
31. A transgenic crop plant comprising a recombinant DNA molecule of a site-specific recombination system resistant to further recombinase activity.

32. A transgenic crop plant of claim 30 or 31 comprising corn, wheat, rice, soybean, potato, canola and cotton.

33. A transgenic potato plant consisting of pMON38267 plant expression cassettes inserted in the genome.

34. A transgenic potato plant consisting of pMON54017 plant expression cassettes inserted in the genome.

35. A DNA cloning kit comprising the methods of claim 17 or 23.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DraI 6695
HindIII 6732
SacI 6753
DraI 4940
DraI 4921
aad
RB
P-FMV
DraI 7422
L-Ph.Hsp70
At.EPSPS:CTP2
ori-322
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CP4syn
rop
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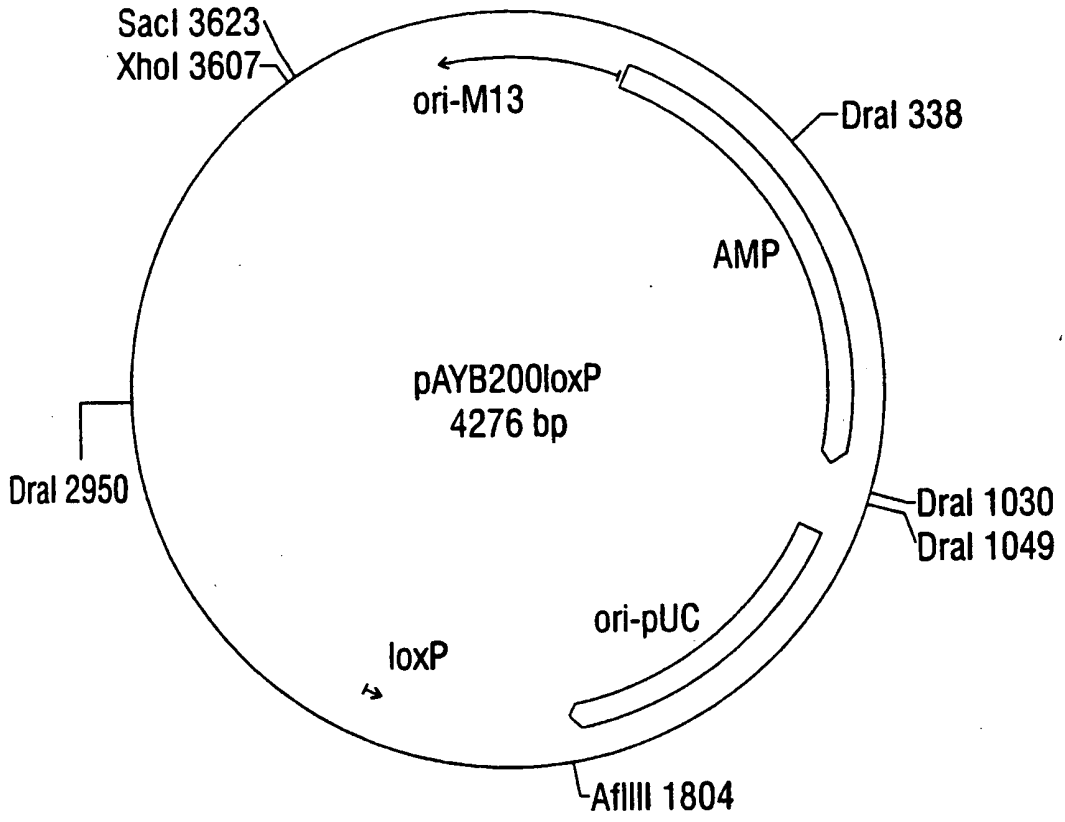
SacI 866
LB
ori-V
EcoRV 1136
DraI 1796

FIG. 1


SacI 3623
XhoI 3607
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DraI 338
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AflIII 1804

FIG. 2

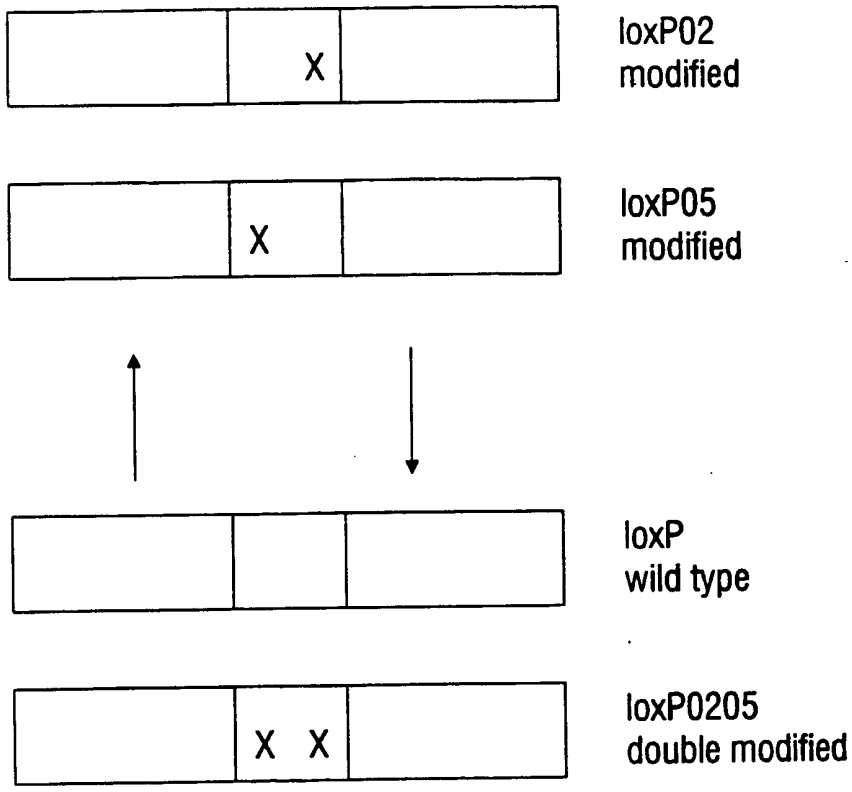

X
loxP02
modified
X
loxP05
modified
loxP
wild type
X X
loxP0205
double modified

FIG. 3

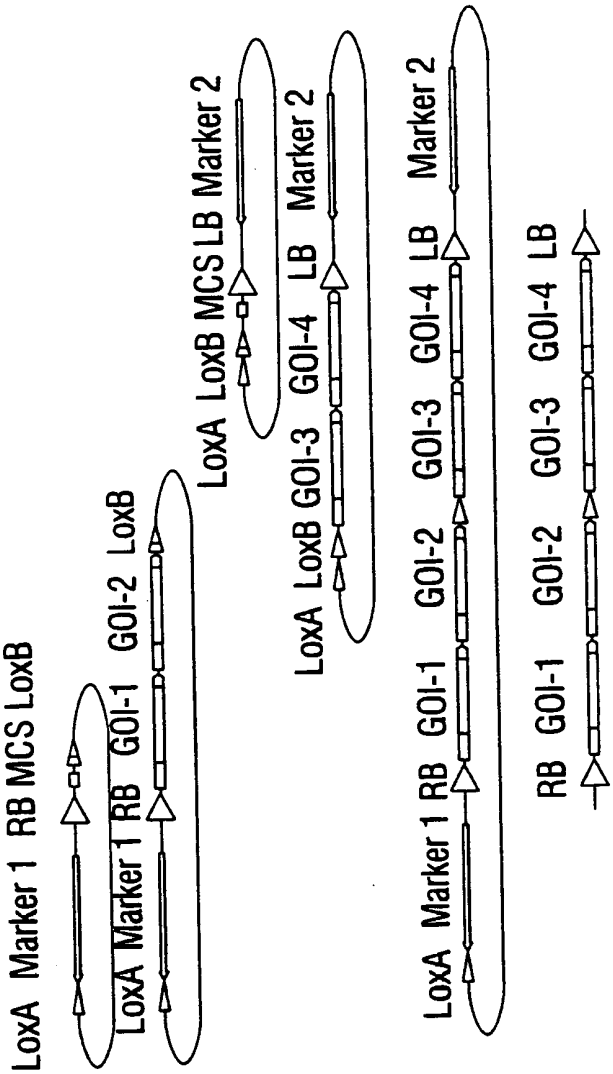
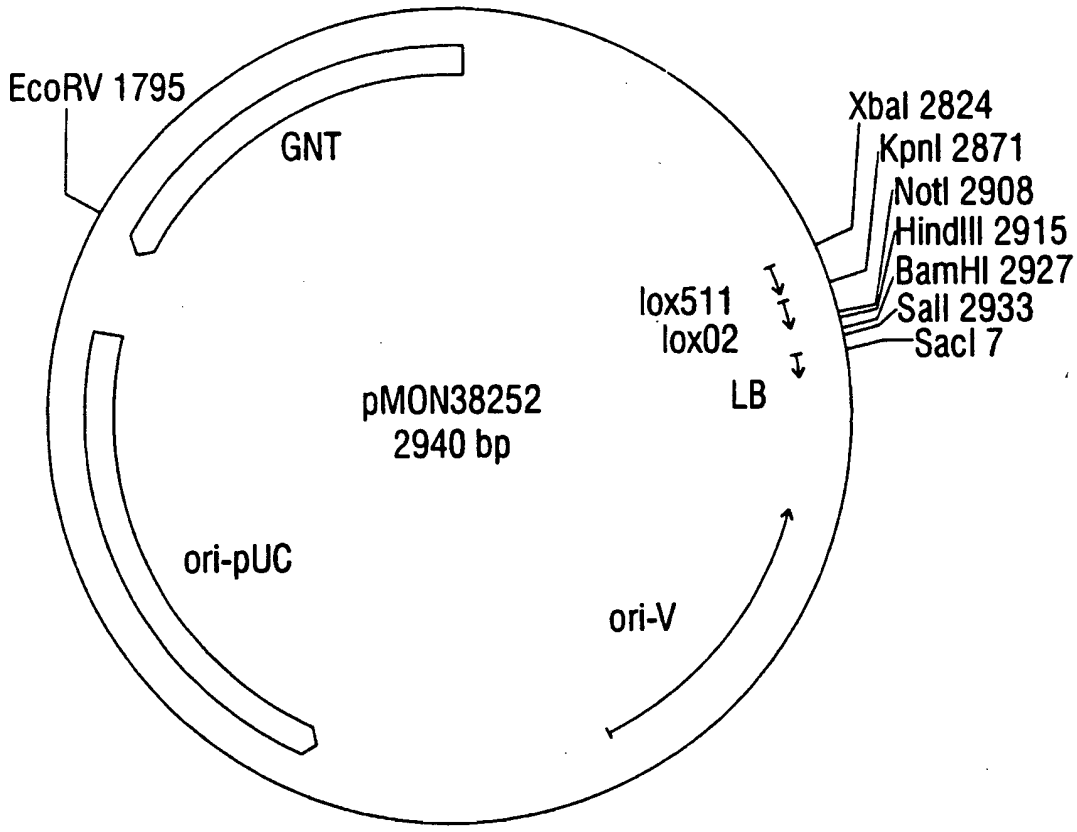

LoxA Marker 1 RB MCS LoxB
LoxA Marker 1 RB GOI-1 GOI-2 LoxB
LoxA LoxB MCS LB Marker 2
LoxA LoxB GOI-3 GOI-4 LB Marker 2
LoxA Marker 1 RB GOI-1 GOI-2 GOI-3 GOI-4 LB Marker 2
RB GOI-1 GOI-2 GOI-3 GOI-4 LB

FIG. 4


EcoRV 1795
GNT
XbaI 2824
KpnI 2871
NotI 2908
HindIII 2915
BamHI 2927
SalI 2933
SacI 7
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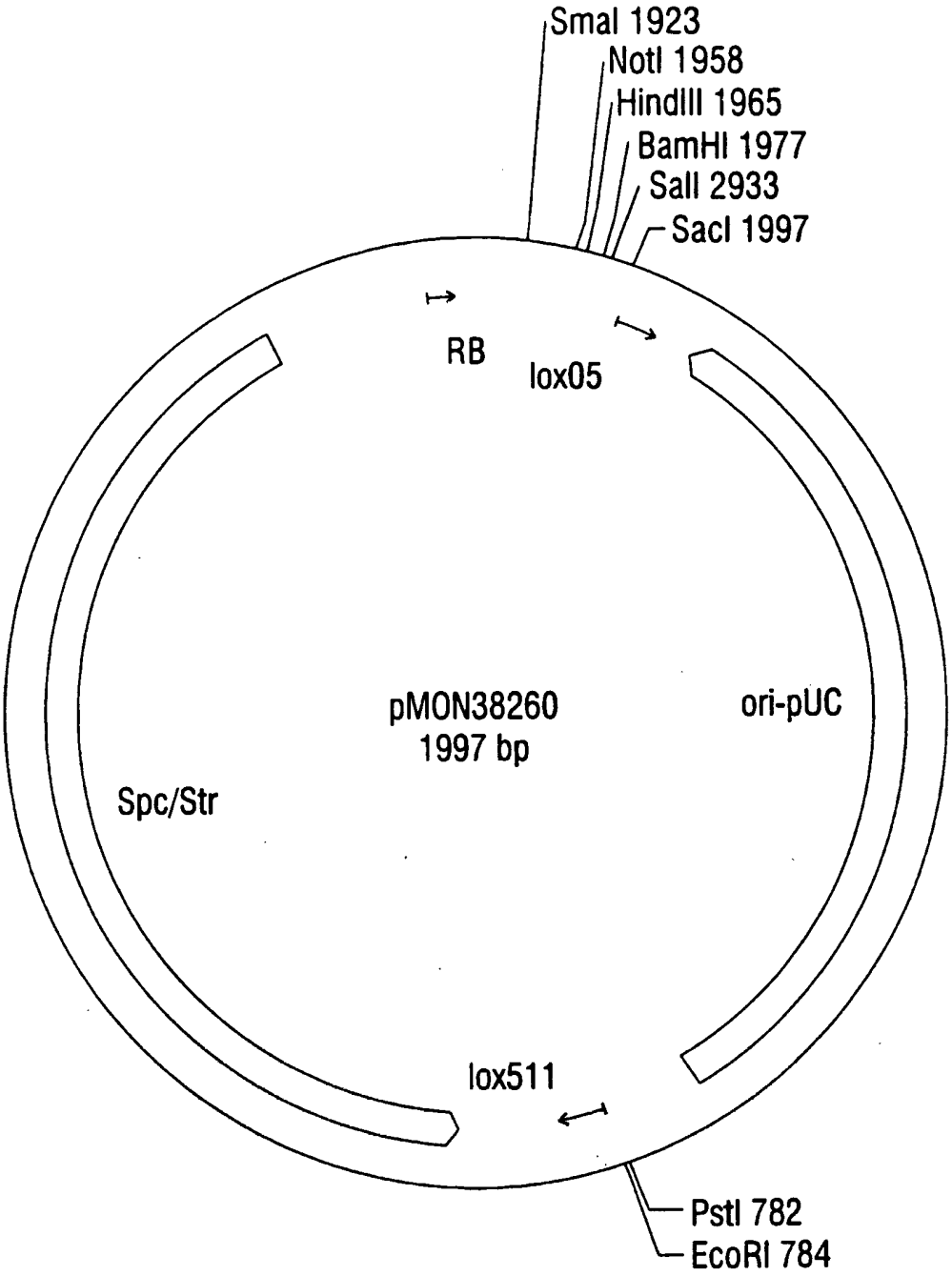
lox02
LB
pMON38252
2940 bp
ori-pUC
ori-V

FIG. 5A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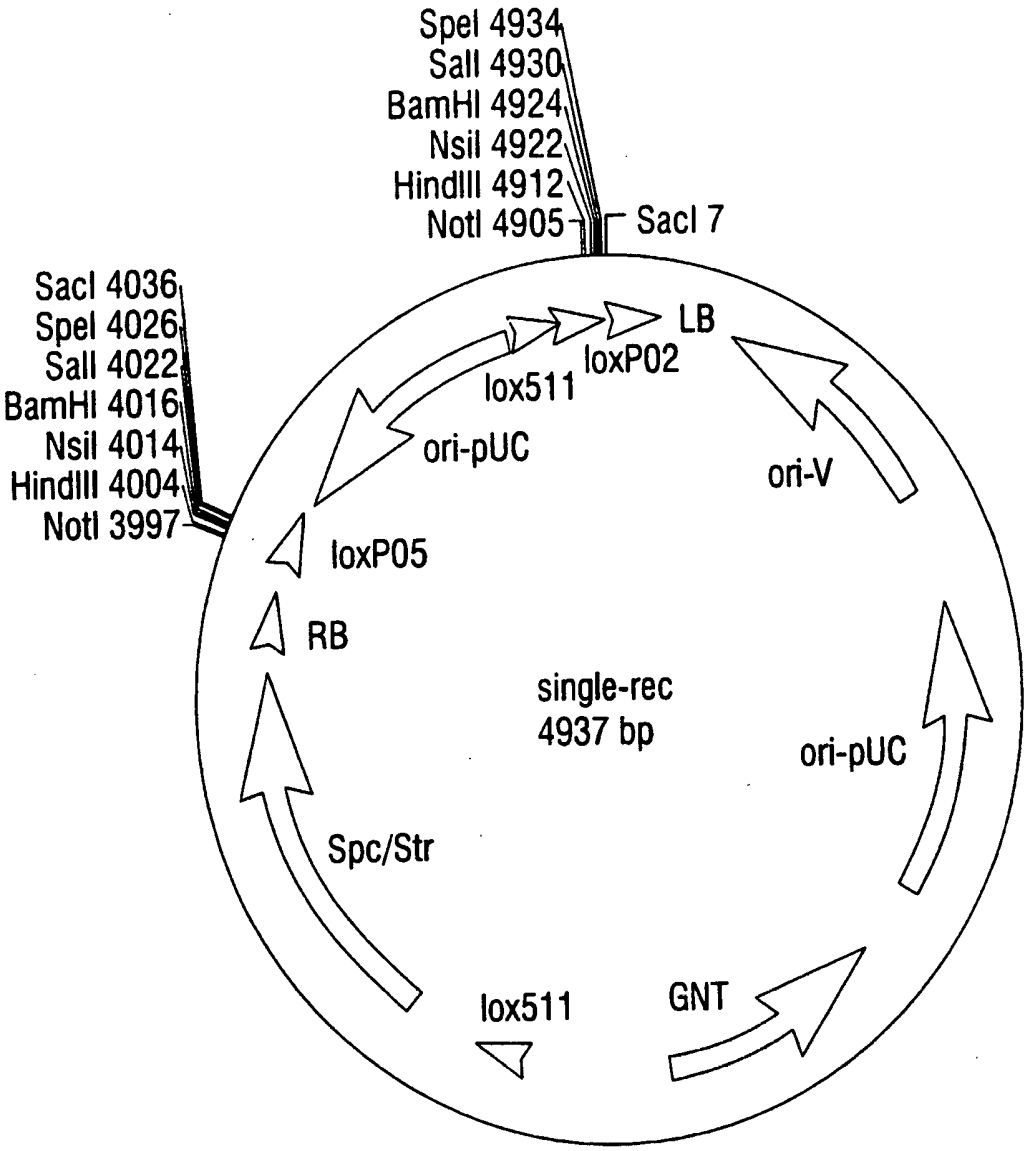
Spc/Str
lox511
PstI 782
EcoRI 784

FIG. 5B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NsiI 4922
HindIII 4912
NotI 4905
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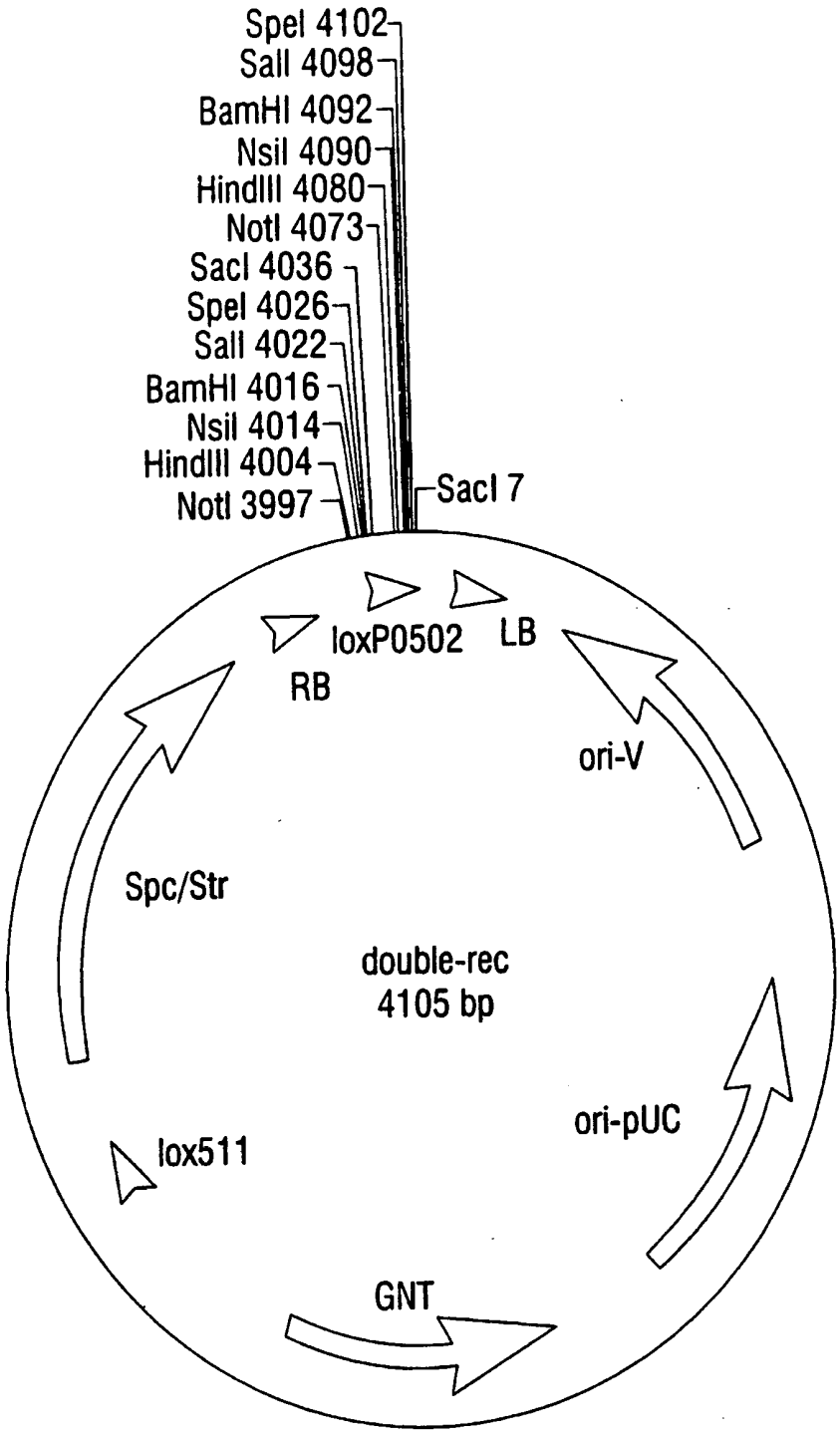
SacI 4036
SpeI 4026
SalI 4022
BamHI 4016
NsiI 4014
HindIII 4004
NotI 3997
LB
loxP02
lox511
ori-pUC
ori-V
loxP05
RB
single-rec
4937 bp
ori-pUC
Spc/Str
lox511
GNT

FIG. 6A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SalI 4098
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NsiI 4090
HindIII 4080
NotI 4073
SacI 4036
SpeI 4026
SalI 4022
BamHI 4016
NsiI 4014
HindIII 4004
NotI 3997
SacI 7
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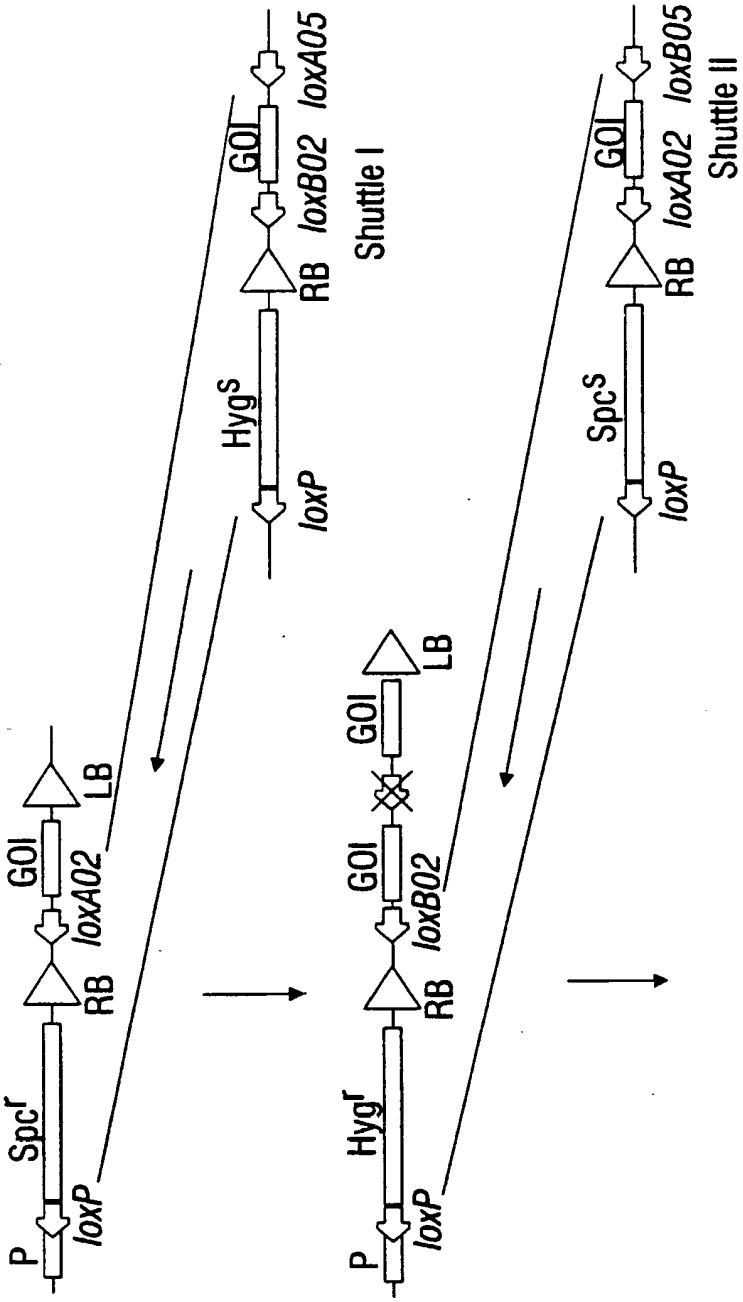
LB
RB
ori-V
Spc/Str
double-rec
4105 bp
ori-pUC
lox511
GNT

FIG. 6B


GOI
loxA05
loxB02
RB
Hyg^S
loxP
Shuttle I
LB
GOI
loxA02
RB
Spc^r
P
loxP
GOI
loxB02
RB
Hyg^r
P
loxP
LB
GOI
loxB05
loxA02
RB
Spc^S
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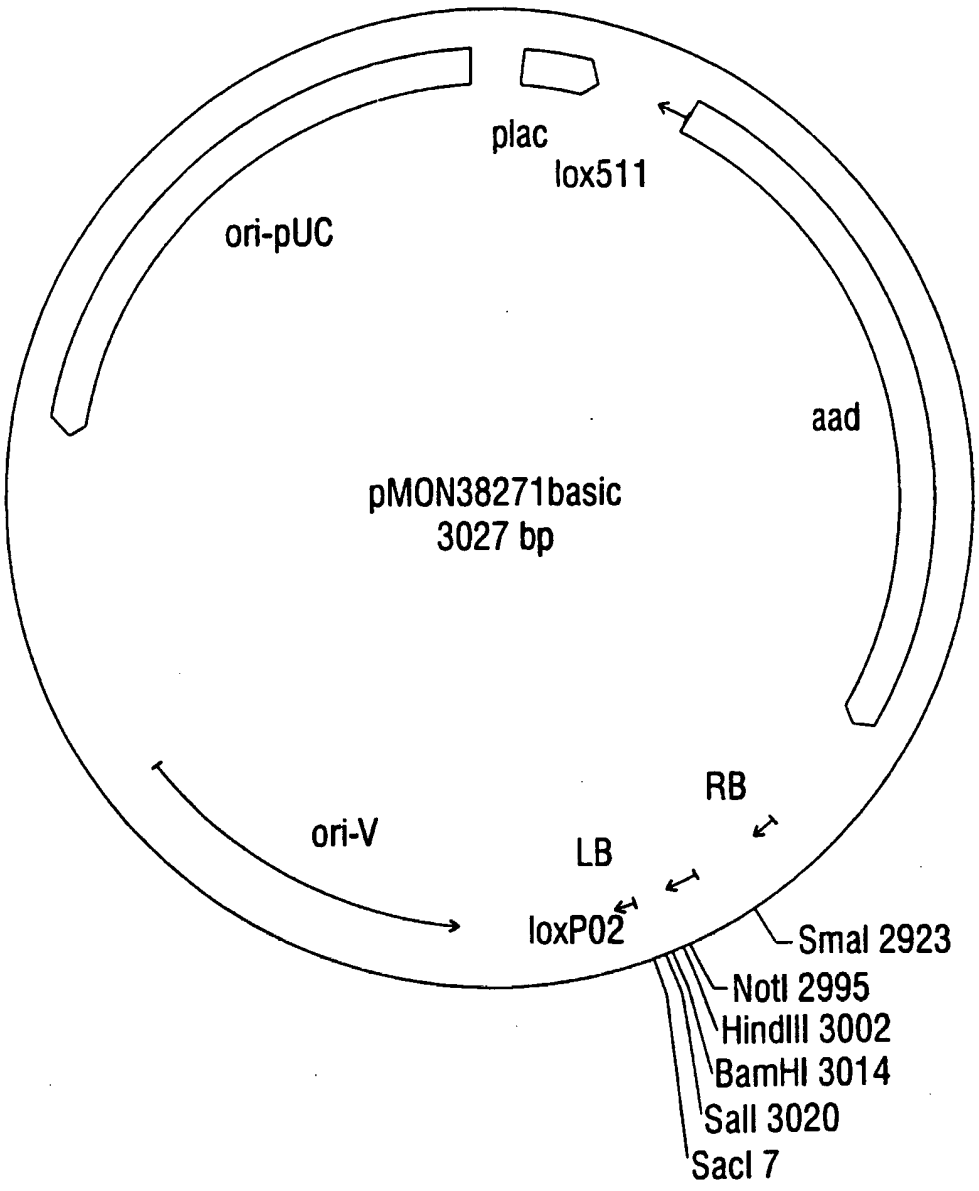
Shuttle II
LB
GOI
GOI
GOI
loxA02
RB
Spc^r
P
loxP

FIG. 7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SmaI 2923
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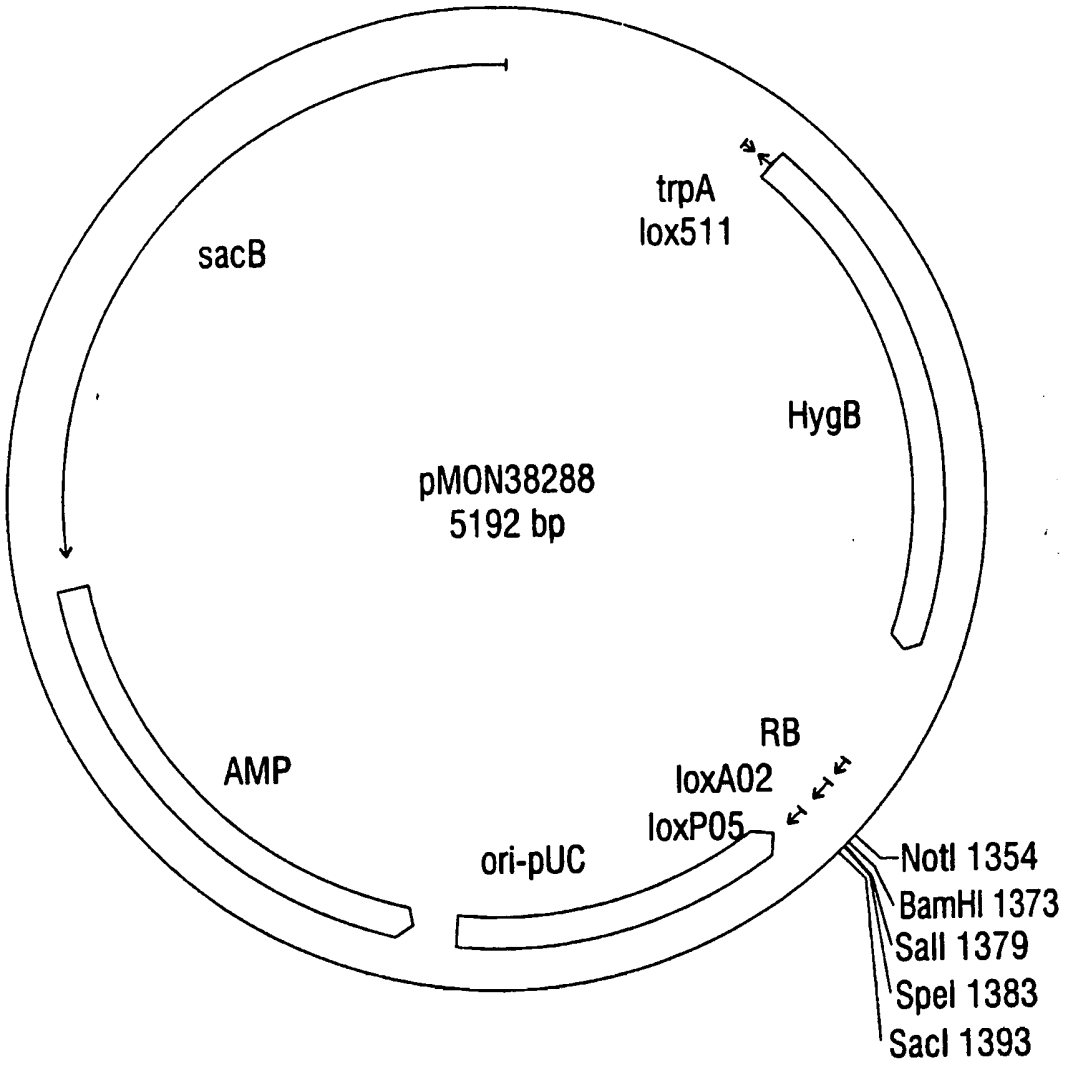
BamHI 3014
SalI 3020
SacI 7

FIG. 8A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lox511
sacB
HygB
pMON38288
5192 bp
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SalI 1379
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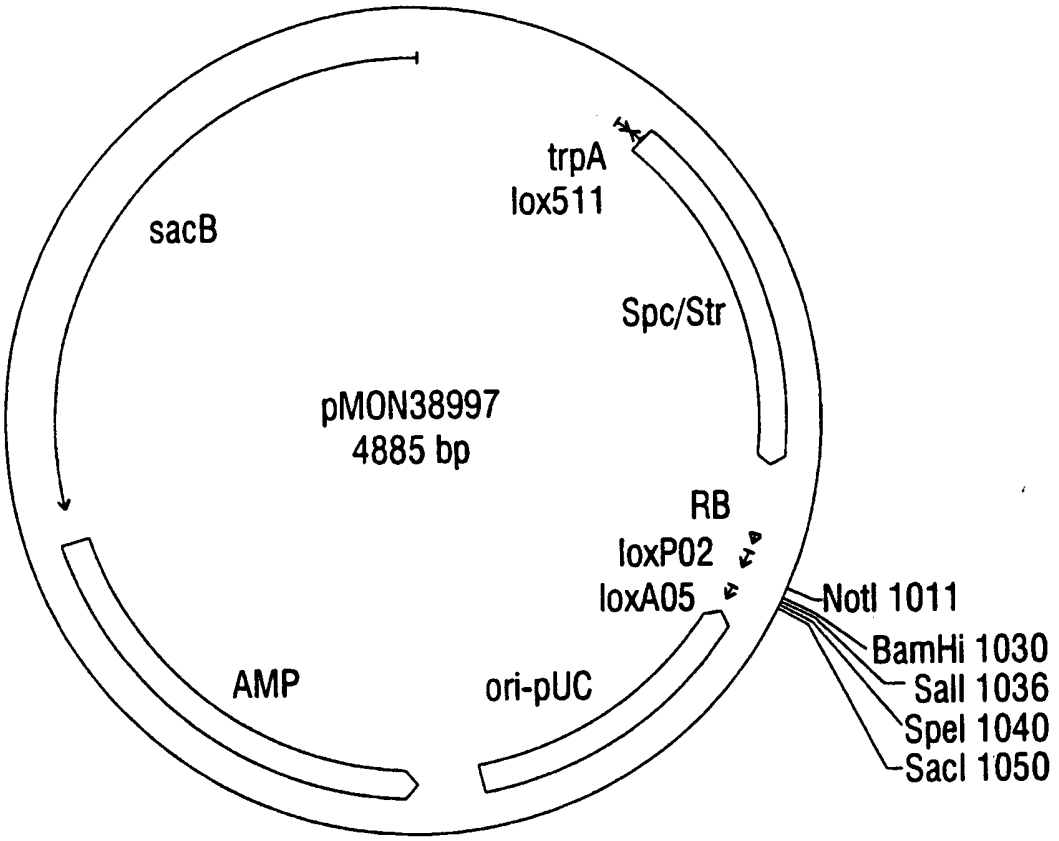
FIG.8B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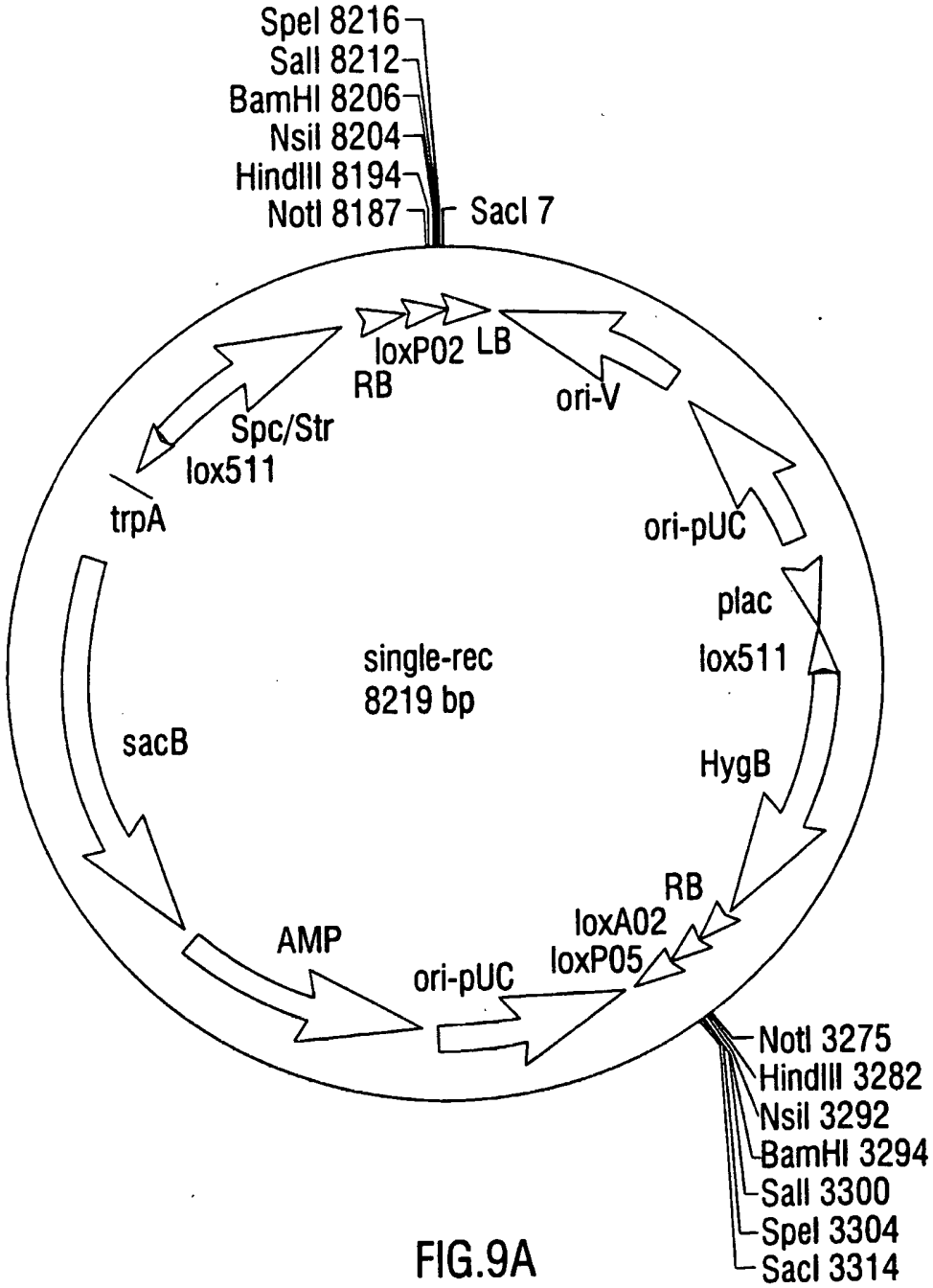
SalI 1036
SpeI 1040
SacI 1050
AMP
ori-pUC

FIG.8C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NsiI 8204
HindIII 8194
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lox511
trpA
ori-pUC
plac
lox511
single-rec
8219 bp
sacB
HygB
RB
loxA02
loxP05
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SalI 3300
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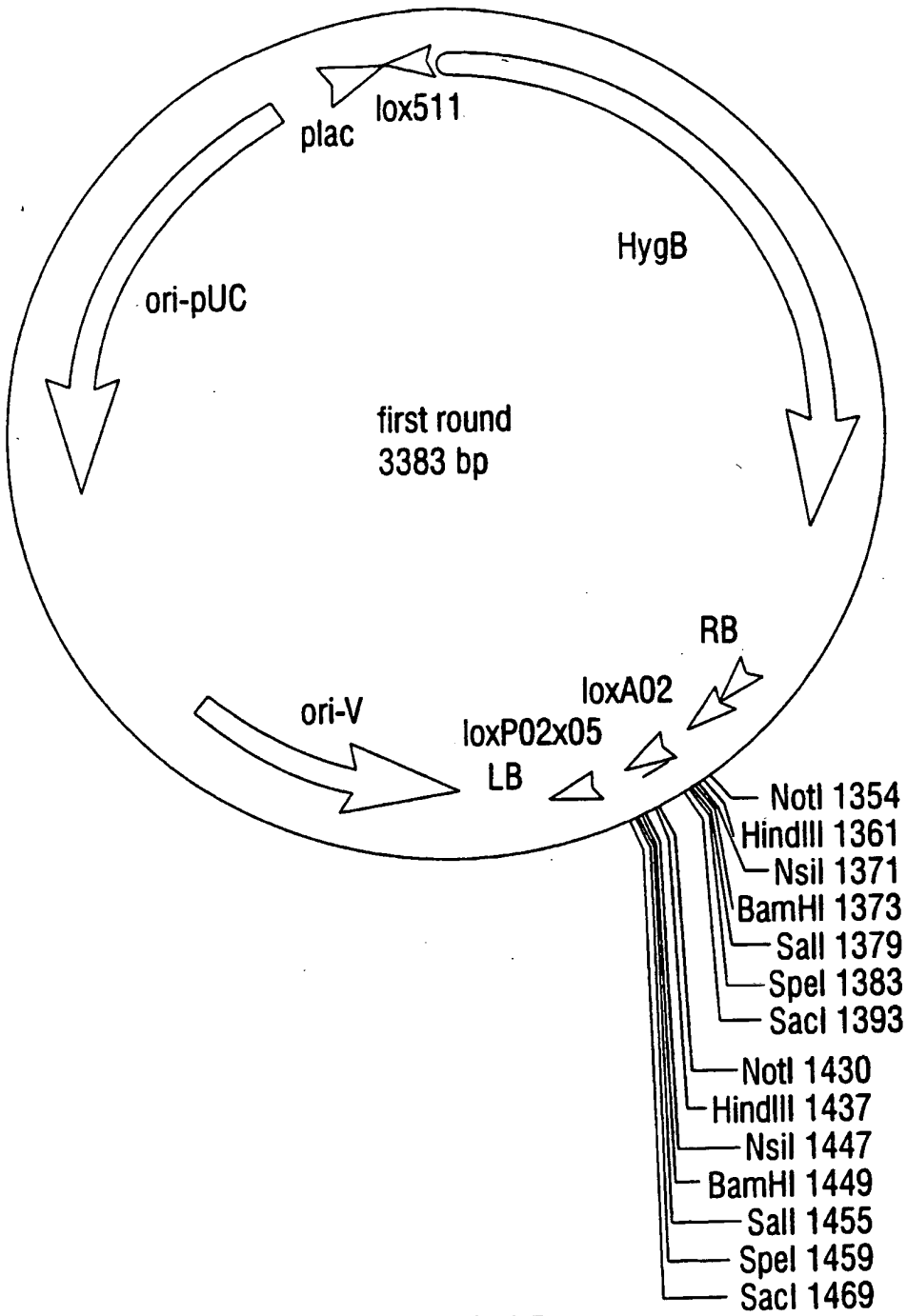
SacI 3314

FIG.9A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HygB
ori-pUC
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3383 bp
RB
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ori-V
loxP02x05
LB
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HindIII 1361
NsiI 1371
BamHI 1373
SalI 1379
SpeI 1383
SacI 1393
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HindIII 1437
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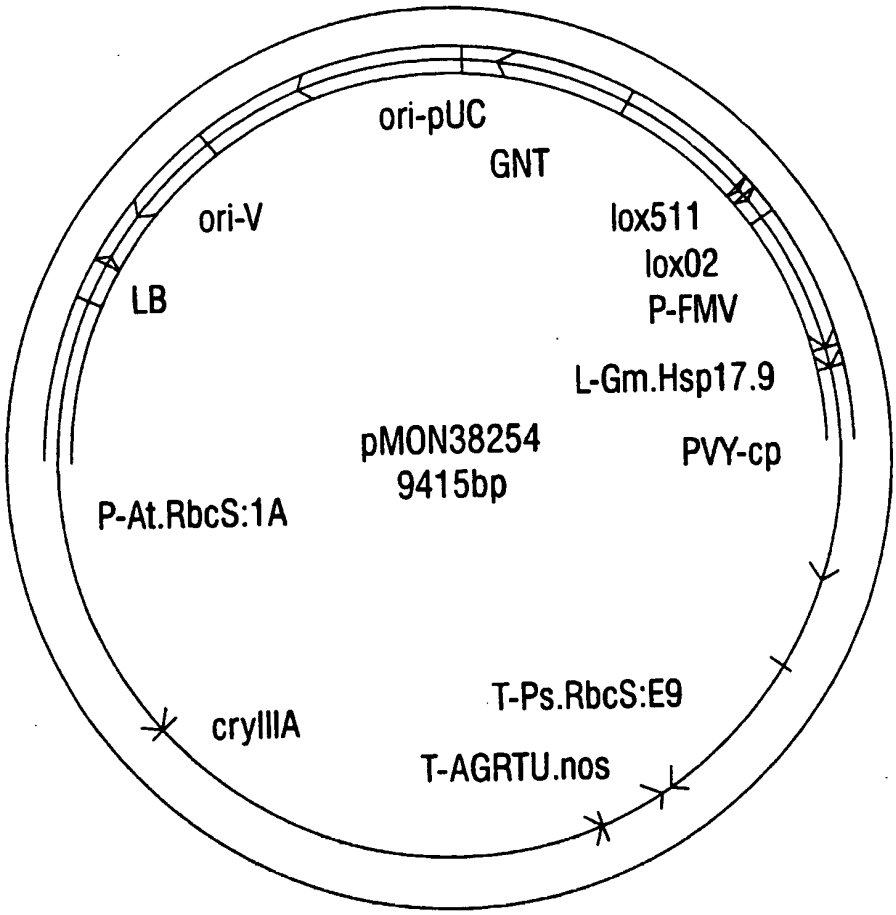
SalI 1455
SpeI 1459
SacI 1469

FIG.9B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ori-V
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lox02
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FIG. 10

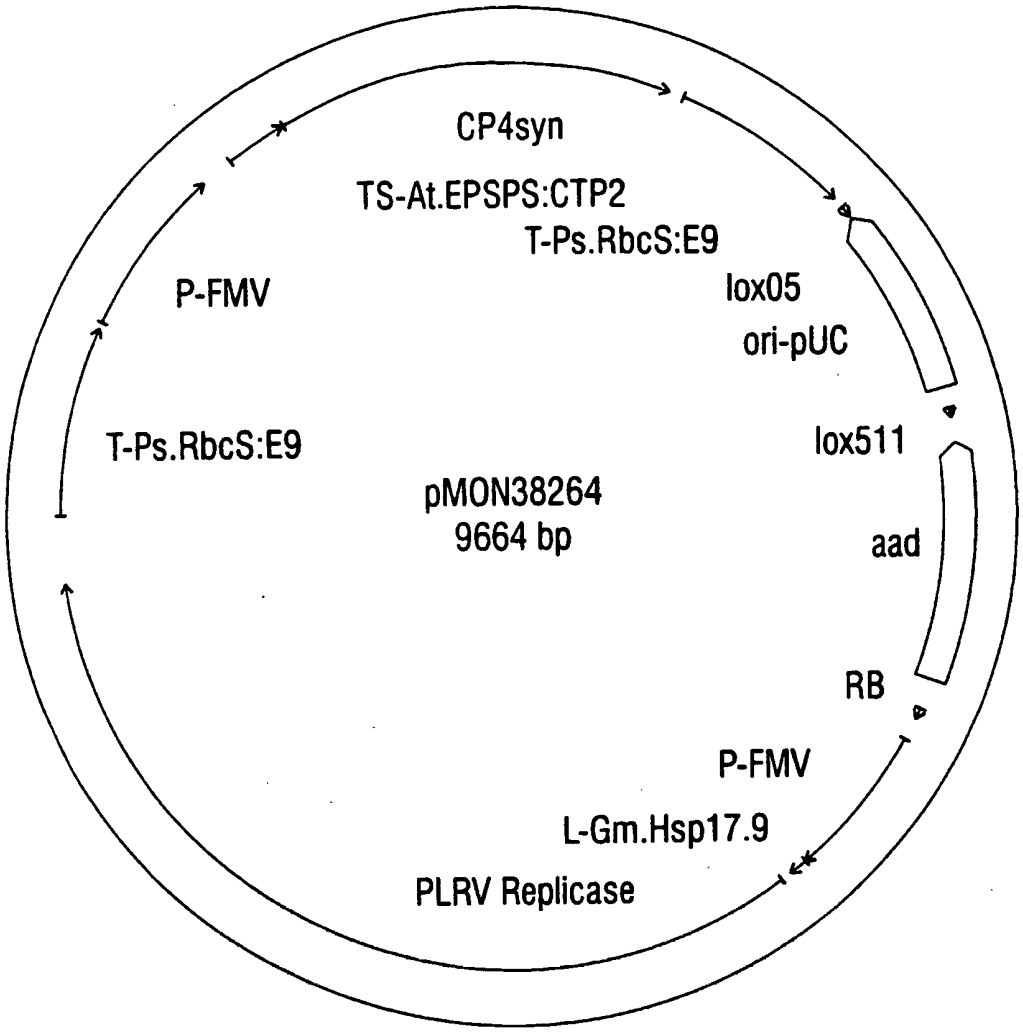
CP4syn
TS-At.EPSPS:CTP2
T-Ps.RbcS:E9
lox05
ori-pUC
lox511
aad
RB
P-FMV
L-Gm.Hsp17.9
PLRV Replicase
T-Ps.RbcS:E9
P-FMV
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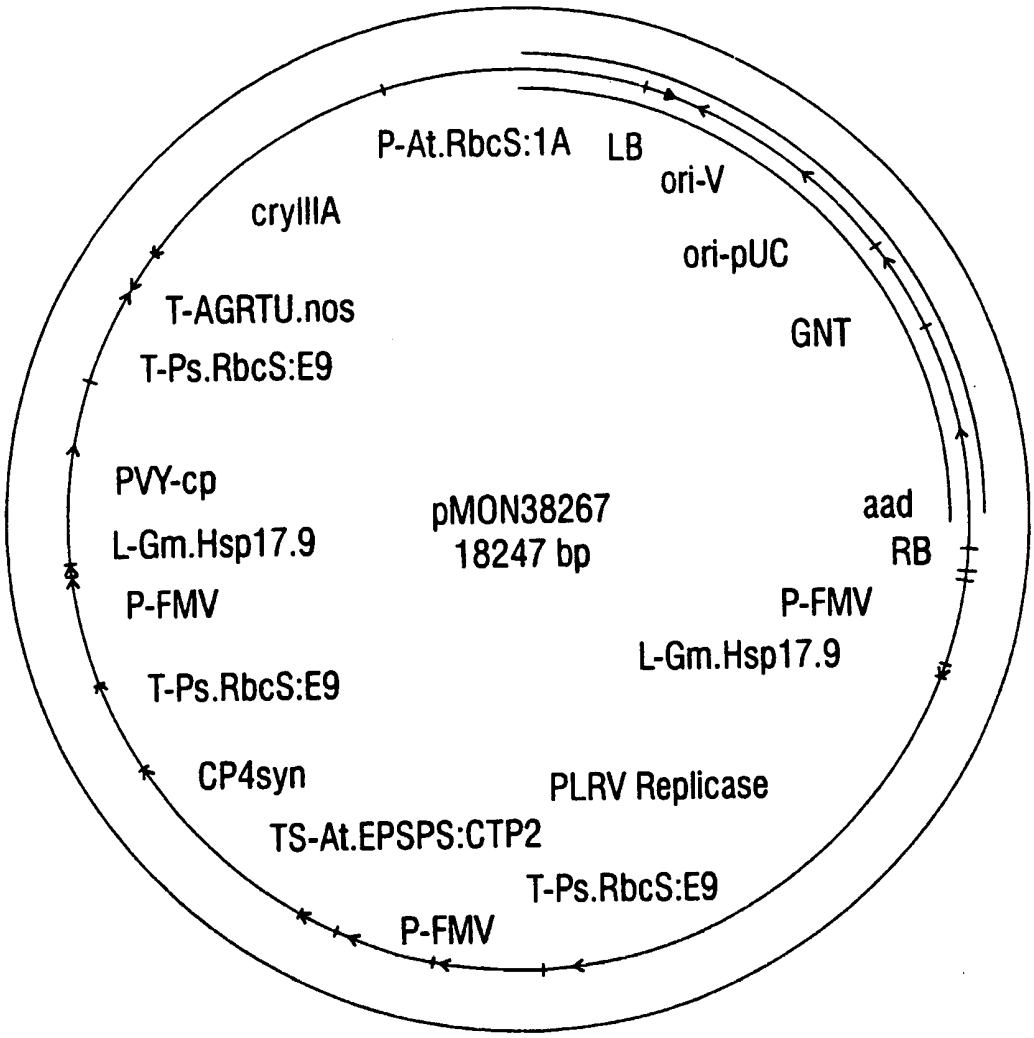
FIG.11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LB
ori-V
cryIIIA
ori-pUC
T-AGRTU.nos
GNT
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PVY-cp
pMON38267
18247 bp
aad
L-Gm.Hsp17.9
RB
P-FMV
P-FMV
L-Gm.Hsp17.9
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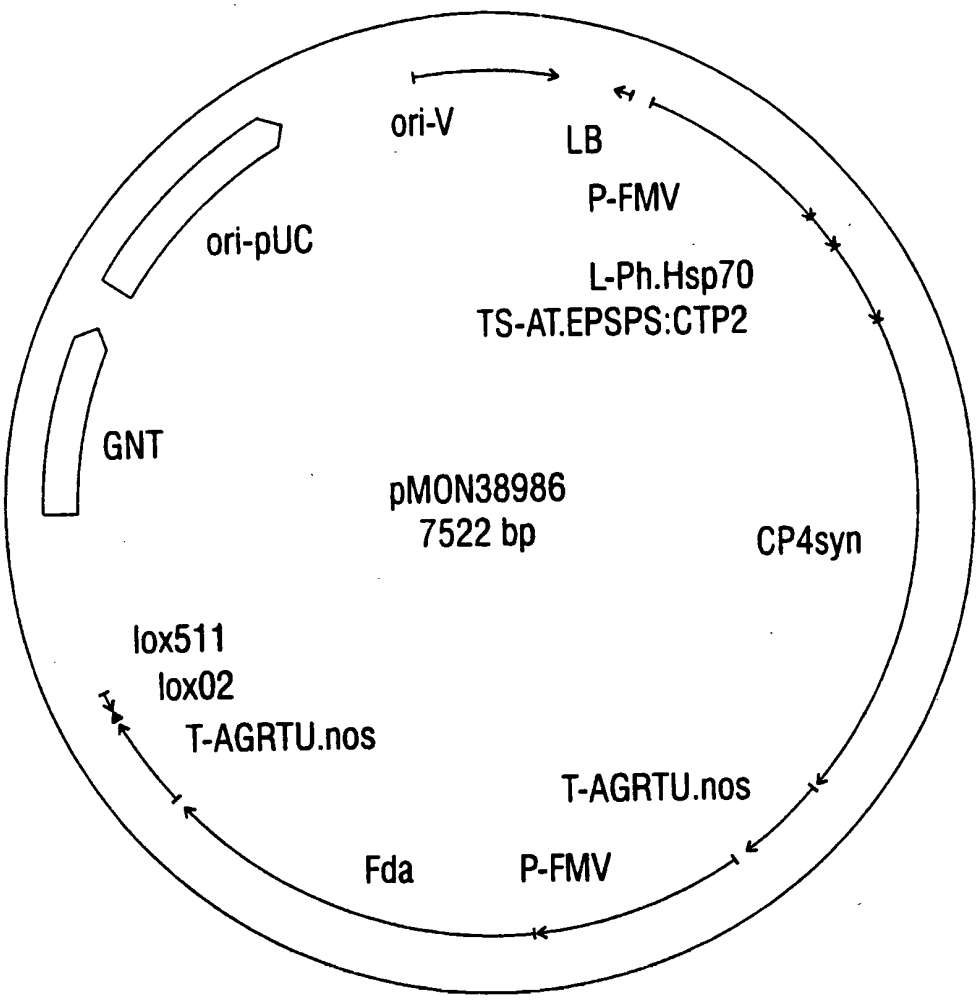
CP4syn
PLRV Replicase
TS-At.EPSPS:CTP2
T-Ps.RbcS:E9
P-FMV

FIG.12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ori-pUC
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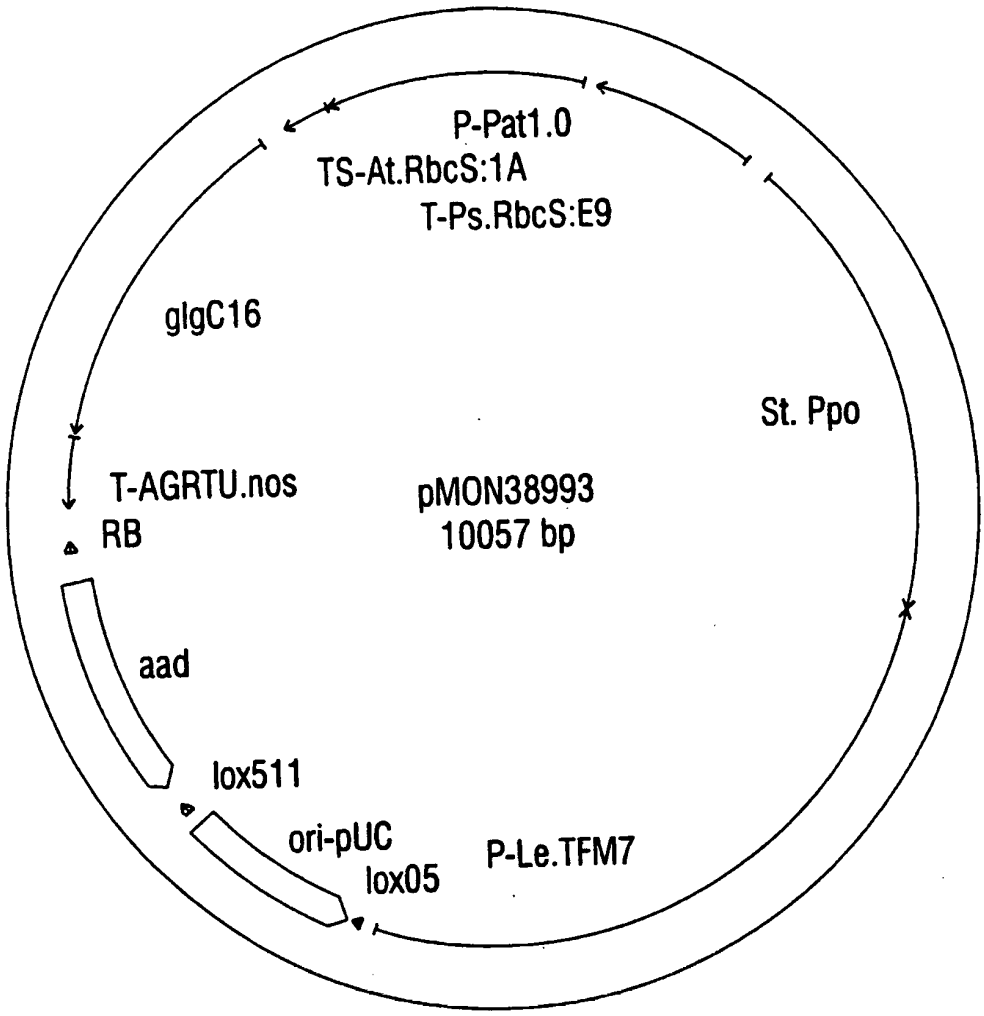
lox511
lox02
T-AGRTU.nos
T-AGRTU.nos
Fda
P-FMV

FIG.13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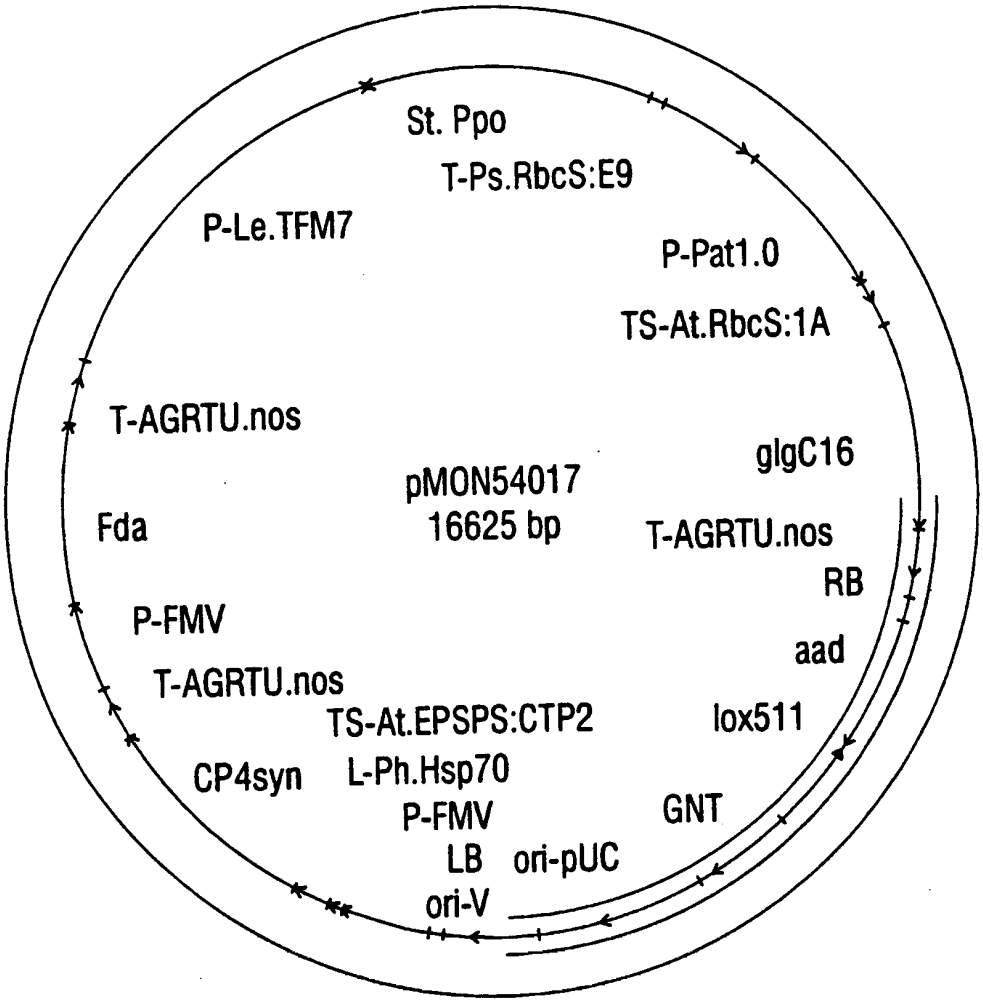
lox05
P-Le.TFM7

FIG.14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P-Le.TFM7
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RB
P-FMV
aad
T-AGRTU.nos
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CP4syn
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ori-pUC
ori-V

FIG.15

SEQUENCE LISTING

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Valentin, Henry E

<120> Novel Cloning Methods

<130> modified site-specific cloning application

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<170> PatentIn Ver. 2.0

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<211> 34
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<220>

<223> Description of Artificial Sequence: synthetic DNA

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<220>
<223> Description of Artificial Sequence: synthetic DNA

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<210> 9
<211> 34
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INTERNATIONAL SEARCH REPORT

Inte Application No
PCT/US 00/21623

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/66 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, BIOSIS, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEE G ET AL: "Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination" GENE,NL,ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, vol. 216, no. 1, August 1998 (1998-08), pages 55-65, XP004149281 ISSN: 0378-1119	3,4,16
Y	the whole document	1,2,5-8, 17-35

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 November 2000	01/12/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bilang, J

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INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 00/21623

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DALE E C ET AL: "MUTATIONS IN THE CRE-LOX RECOMBINATION SITE ENHANCE THE STABILITY OF RECOMBINATION PRODUCTS APPLICATIONS FOR GENE TARGETING IN PLANTS" JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT, no. 16 PART F, 1992, page 206 XP000942839 ISSN: 0733-1959 the whole document ---	1-35
Y	SCHLAKE T ET AL: "USE OF MUTATED FLP RECOGNITION TARGET (FRT) SITES FOR THE EXCHANGE OF EXPRESSION CASSETTES AT DEFINED CHROMOSOMAL LOCI" BIOCHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. EASTON, PA, vol. 33, no. 43, 1 November 1994 (1994-11-01), pages 12746-12751, XP000616165 ISSN: 0006-2960 the whole document ---	1-35
Y	BETHKE B ET AL: "SEGMENTAL GENOMIC REPLACEMENT BY Cre-MEDIATED RECOMBINATION: GENOTOXIC STRESS ACTIVATION OF THE p53PROMOTER IN SINGLE-COPY TRANFORMANTS" NUCLEIC ACIDS RESEARCH,OXFORD UNIVERSITY PRESS, SURREY,GB, vol. 25, no. 14, 1997, pages 2828-2834, XP002122405 ISSN: 0305-1048 the whole document ---	1,2, 5-15, 17-35
Y	ALBERT H ET AL: "Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome" PLANT JOURNAL,GB,BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, vol. 7, no. 4, 1995, pages 649-659, XP002097329 ISSN: 0960-7412 the whole document ---	9-15
Y	SAUER B: "MULTIPLEX CRE/IOX RECOMBINATION PERMITS SELECTIVE SITE-SPECIFIC DNATARGETING TO BOTH A NATURAL AND AN ENGINEERED SITE IN THE YEAST GENOME" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 24, no. 23, 1996, pages 4608-4613, XP002913183 ISSN: 0305-1048 the whole document --- -/--	17-35

INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/US 00/21623

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 25851 A (SAITO IZUMU ;SUMITOMO PHARMA (JP); TANAKA KEIJI (JP)) 27 May 1999 (1999-05-27) -& EP 1 035 208 A (SUMITOMO PHARMACEUTICALS COMPANY LDT) 13 September 2000 (2000-09-13) the whole document	
P,X	WO 99 55851 A (NOVARTIS ERFINDUNGEN VERWALTUN ;NOVARTIS AG (CH); CHILTON MARY DEL) 4 November 1999 (1999-11-04) the whole document	1-35

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/21623

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9925851 A	27-05-1999	JP 11196880 A	27-07-1999
		AU 1052699 A	07-06-1999
		EP 1035208 A	13-09-2000
WO 9955851 A	04-11-1999	AU 3928899 A	16-11-1999